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Newsletter for the Neuroscientist

Protein Kinase Substrates Available from RBI


Substrates

RBI offers peptide substrates for a range of protein kinases, including Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase), cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and tyrosine kinases. These substrates are synthetic peptides, are greater than 96% pure and are available now. Look for the introduction of more protein kinase substrates in future issues of *Neurotransmissions*.

Ca^{2+} /Calmodulin-Dependent Kinases

- C-183 CaM Kinase II substrate
Substrate for Ca^{2+} /calmodulin-dependent kinase II
H-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser-NH₂
- A0591 CaM Kinase II selective substrate (Autocamtide-2)
Highly selective substrate for Ca^{2+} /calmodulin-dependent kinase II
Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Thr-Val-Asp-Ala-Leu
- C-229 CaM Kinase IV substrate (Peptide-gamma)
Substrate for Ca^{2+} /calmodulin-dependent kinase IV
Lys-Ser-Asp-Gly-Gly-Val-Lys-Lys-Arg-Lys-Ser-Ser-Ser-Ser

cAMP-Dependent Protein Kinase

- K1127 Kemptide
Substrate for cAMP-dependent protein kinase (PKA)
 Leu-Arg-Arg-Ala-Ser-Leu-Gly
- P-177 Protein kinase A (PKA)
Substrate for protein kinase A
H-Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg-OH
- A3317 Malantide
cAMP-dependent protein kinase (PKA) and protein kinase C substrate
Arg-Thr-Lys-Arg-Ser-Gly-Ser-Val-Tyr-Glu-Pro-Leu-Lys-Ile

Protein Kinase C

- M-215 MARCKS protein [151-175] (Myristoylated alanine-rich C kinase substrate protein [151-175]) High affinity protein kinase C substrate
Lys-Lys-Lys-Lys-Lys-Arg-Phe-Ser-Phe-Lys-Lys-Ser-Phe-Lys-Leu-Ser-Gly-Phe-Ser-Phe-Lys-Lys-Asn-Lys-Lys
- M-214 MARCKS protein [159-165] (Myristoylated alanine-rich C kinase substrate protein [159-165]) High affinity protein kinase C substrate
Phe-Lys-Lys-Ser-Phe-Lys-Leu
- P2186 Protein kinase C selective substrate ([pGlu⁴] (4-14) myelin basic protein
pGlu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu-OH
- P-174 Protein kinase C substrate
Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu
- P-212 [Ser²⁵]-Protein kinase C (19-36) substrate
High affinity protein kinase C pseudosubstrate
Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn
- ### Tyrosine Kinases
- A7433 Insulin receptor tyrosine kinase substrate
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly
- A7907 Tyrosine kinase substrate
H-Arg-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gly-OH

Protein Kinase Antibodies Available from RBI

RBI offers antibodies to a variety of protein kinases. See page 17 for our newest antibodies to MAP kinases and PKC isoforms.

Chemokines and CNS Inflammation

Richard M. Ransohoff

Chemokines are a group of small (8-10 kDa), secreted proteins that were initially identified by their ability to act as leukocyte chemoattractants. As *chemoattractant cytokines*, these molecules induce leukocytes to migrate along concentration gradients, and modulate leukocyte-endothelial interactions through the upregulation and reversible activation of leukointegrins. In contrast to leukotrienes or complement components, chemokines are cell-type selective chemoattractants. In this sense, chemokines are proposed to define the cellular composition of inflammatory infiltrates. However, recent evidence suggests that, in addition to functioning as important regulators of the immune system, these molecules also play important roles within the circulatory and central nervous systems (CNS).

Inflammatory stimuli lead to upregulation of the genes for both the chemokines and their receptors, which has permitted their efficient cloning using differential display technologies. Because the gene products have been initially characterized as attractants and activators of leukocyte subpopulations *in vitro*, there is a daunting impression of chemokine functional overlap and redundancy. Thus, this approach to characterizing new chemokines gives a falsely limited view of their spectrum of functions. Importantly, apparently functionally redundant chemokines are differentially regulated, giving rise to specific roles *in vivo* during inflammation

and host defense mechanisms. The immense accumulation of knowledge about this recently described gene family has been fueled by the fact that these proteins are implicated in a vast array of important pathological states, including atherosclerosis, cancer, AIDS and CNS diseases.

Classification of chemokines

Chemokine genes and their gene products have been described in humans, non-human primates, guinea-pigs, rats, rabbits and mice and, to date, more than 40 chemokines have been identified. With the exception of lymphotactin, all chemokines contain at least four cysteine residues in nearly invariant positions. They can be divided into four subfamilies, based on structural, functional and genetic criteria [1-4]. One major chemokine subfamily is the CXC, or α -family, in which two N-terminal positionally-conserved cysteine residues are separated by a single amino acid (see Figure 1). The prototype α -chemokines, such as IL-8 (interleukin-8), are neutrophil-directed and possess a conserved glutamate-leucine-arginine (ELR) motif in the N-terminal receptor-binding domain. Non-ELR α -chemokines, such as IP-10 (γ -interferon inducible protein), MIG (monokine induced by γ -interferon) and β -R1/I-TAC, act as chemoattractants for activated T cells and have no effect on neutrophils.

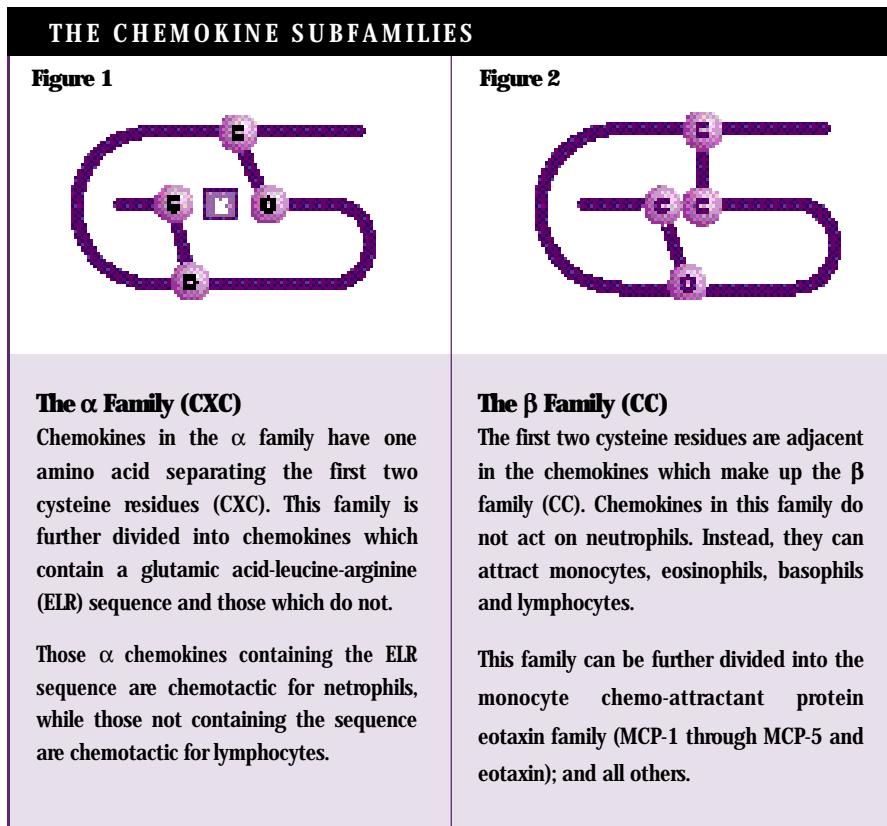
About the Author

Richard Ransohoff received his M.D. degree from Case Western Reserve University, Ohio followed by residences at Mount Sinai Medical Center, New York and the Cleveland Clinic, Ohio. After a period of post-doctoral research in the Department of Molecular Biology and Microbiology at Case Western Reserve University School of Medicine, he joined the Cleveland Clinic Foundation eventually reaching the positions of Staff Scientist within the Department of Neurosciences at the Lerner Research Institute and Staff Neurologist at the Mellen Center for Multiple Sclerosis Treatment and Research. In 1997, he was appointed to the rank of Professor within the Department of Medical Microbiology and Immunology at the Cleveland Health Sciences Center of Ohio State University.

Chemokines and CNS Inflammation (cont.)

A second major chemokine subfamily is the CC or β -family, in which the two N-terminal cysteines are adjacent to each other (see Figure 2). Chemokines in this family do not act on neutrophils, and instead attract monocytes, eosinophils, basophils and lymphocytes. Members of this family can be further divided into two groups: the monocyte chemoattractant protein/eotaxin family (MCP-1 through MCP-5 and eotaxin) and all others. In humans, CXC chemokines are encoded on chromosome 4, while many CC chemokines are found on chromosome 17. However, more recently identified members of the human CC chemokine family are encoded outside these loci.

Lymphotactin, a lymphocyte chemoattractant, belongs to a third family of chemokines [5,6]. It is structurally and functionally similar to traditional chemokines, but lacks two of the four hallmark cysteine residues and is encoded outside the chemokine loci on human chromosome 1. This family has been termed the C or γ family (see Figure 3). Lastly, 'neurotactin' or 'fractalkine,' is the index member of a family bearing an N-terminal CXXXC (CX_3C) motif. (see Figure 4) Interestingly, neurotactin/fractalkine was initially isolated by Pan *et al.* from a choroid plexus cDNA library, and is highly expressed in brain and upregulated by inflammatory processes [7].

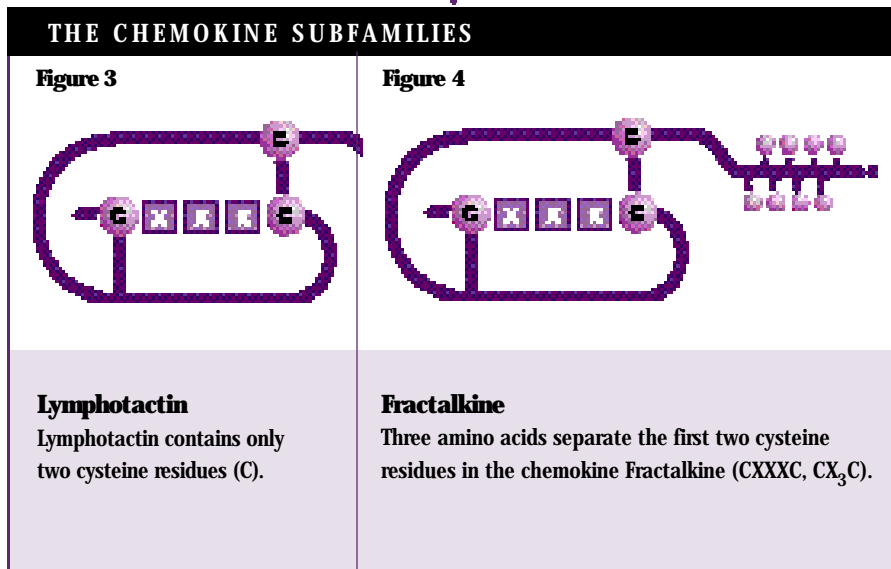


Chemokine receptors and signaling

Chemokine responses depend on the expression and activation of cell surface receptors, which belong to the family of G protein-coupled receptors. The receptors that respond to α -chemokines and β -chemokines differ structurally, and are typically encoded by genes that possess intronless open reading frames. There are at present five cloned receptors that respond to α -chemokines (CXCR1-5), nine cloned human receptors that respond to defined β -chemokines (CCR1-9), and one receptor for neurotactin/fractalkine (CX₃CR1). Interestingly, it was found that both the rat and human CX₃CR1 receptors had been previously isolated as orphan receptors that are highly expressed in normal adult brain [8,9]. As multiple chemokines can bind productively to a single receptor and multiple receptors bind individual chemokines [10,11], the relationship between chemokines and their receptors has been termed promiscuous.

While this can be confusing with respect to the physiological relevance of these interactions, cells capable of responding to individual chemokines can be identified by their pattern of chemokine receptor expression.

Multiple signaling pathways are linked to chemokine receptor activation indicating heterogeneity of receptor coupling mechanisms. Effects can be broadly divided into pertussis toxin-sensitive and pertussis toxin-insensitive responses, based on the susceptibility of the coupled G protein to inhibition by pertussis toxin-catalyzed ADP ribosylation. Pertussis toxin-sensitive responses are mediated through G_i proteins, which effect the inhibition of adenylyl cyclase, thereby leading to decreased cellular concentrations of cAMP. Chemokine signaling is also linked to phospholipase C (PLC) activation and the generation of transient elevations of cytosolic Ca²⁺. At least some of the increases in cytosolic calcium seen in



Chemokines and CNS Inflammation (cont.)

response to chemokine stimulation are thought to be mediated by pertussis toxin-insensitive activation of PLC β through coupling to G_{q/11}-type G proteins. However, activation of this enzyme has also been described as pertussis toxin-insensitive in response to IL-8, implicating the involvement of G_i-type G proteins. Chemokine receptor engagement and G protein activation can also lead to the activation of Jak/STAT pathways, MAP kinase pathways, ARF-dependent phospholipase D activation and RhoA

activation. PKB/akt, which has been implicated in neuronal cell survival, is activated by chemokines in neutrophils and monocytes and is blocked by pertussis toxin treatment. Thus, the specific pathways activated in different cell types by different complements of chemokines and chemokine receptors can produce very specific responses in individual cells. Selective signaling in specific cell types may suggest opportunities for selective pharmacological intervention.

CHEMOKINE PRODUCTS AVAILABLE FROM RBI				
HUMAN recombinant chemokines		ANTI-HUMAN chemokine antibodies	MOUSE recombinant chemokines	RAT recombinant chemokines
Eotaxin	MCP-4	Eotaxin	C10	CINC-1
Eotaxin-2	MDC	GRO α	CRG-2	CINC-2 α
Fractalkine	MIG	IL-8	Eotaxin	CINC-2 β
GCP-2	MIP-1 α	IP-10	GCP-2	MCP-1
GRO α	MIP-1 β	MCP-1	MARC	For additional information on RBI Chemokines, contact our Technical Service Department at 508-651-8151; or fax: 508-655-1359 or e-mail: tech@resbio.com In U.S. and Canada call 800-736-3690 or fax: 800-736-2480
HCC-1	MIP-3 α	MCP-2	MCP-5	
I-309	MIP-3 β	MCP-3	MIP-1 α	
IL-8	NAP-2	MIP-1 α	MIP-1 β	
IP-10	RANTES	MIP-1 β	MIP-1 γ	
MCP-1	SDF-1 α	RANTES	MIP-2	
MCP-2	SDF-1 β	SDF-1 α		
MCP-3				

Studies of chemokine function

Much interest has recently been generated by the observation that certain chemokine receptors (including CCR5, CXCR4, CCR2b and CCR3) are obligate invasion cofactors, along with CD4, for immunodeficiency viruses such as HIV-1, HIV-2 and SIV [12]. A major focus of recent research has been to define susceptibility to HIV invasion in the context of receptor expression. Therefore, chemokine receptor expression by subpopulations of T-cells, monocytes, macrophages, glia and neurons has been intensely studied.

There is also a growing body of literature describing chemokine receptor expression by neuroepithelial cells in the CNS [13-17]. For example, immunohistochemical studies indicate that CXCR4 receptors are expressed by a variety of subpopulations of neurons and glia [15,18]; CXCR2 receptors have been detected on both neurons and oligodendrocyte progenitors [14,19]; and CXCR2 receptors have been shown to be abundant on degenerating neurites in Alzheimer's disease plaques [14,20]. The expression of CCR1 and CXCR4 receptors on astrocytes *in vitro* has also been described [17,21], while CCR3 and CCR5 receptors have been detected on microglia [16]. In some reports, CCR5 receptors have also been described on subpopulations of neurons [13]. More recent observations *in vitro* indicate that chemokine receptor expression is regulated by inflammatory cytokines and *in vivo* that several receptors are upregulated in the inflammatory disease model, experimental autoimmune encephalomyelitis (EAE) [22]. Finally, the Duffy blood group antigen is identical to a promiscuous chemokine binding protein on erythrocytes and postcapillary venules. In its chemokine-binding guise, this molecule is termed the Duffy Antigen Receptor for

Chemokines (DARC), and is detected on Purkinje cells in human cerebellum [23]. Taken together, these varied observations support the hypothesis that a variety of cells in the CNS can express chemokine receptors, and imply a broad role for chemokines in physiological and pathophysiological states in the CNS.

Experiments involving the use of chemokine knockout mice challenge the notion of redundancy and indicate the importance of these animals in investigating the roles of chemokines in disease. For example, mice devoid of macrophage inflammatory protein-1 α (MIP-1 α) exhibit selective failure of antiviral immune defense systems. Thus, they are unable to generate inflammatory responses to pulmonary challenge with influenza virus and do not develop the expected myocarditis after infection with Coxsackie virus [24]. Interestingly, the inflammatory response to influenza is protective, while the response to Coxsackie causes severe cardiomyopathy. Therefore, MIP-1 α knockouts are *less* susceptible to fatal inflammatory cardiomyopathy, but *more* vulnerable to pulmonary influenza [24].

Mice lacking the genes for MCP-1, the CCR2 receptor or the CCR5 receptor exhibit various alterations in leukocyte migration, cytokine production and host responses [25-28]. Importantly, these chemokine and receptor knockouts are fertile and viable, and also exhibit Mendelian inheritance of the nullizygous trait, without gross developmental alteration in circulating or bone marrow leukocyte populations or immune functions. These attributes of the MIP-1 α , MCP-1, CCR5 and CCR2 knockouts arise from the fact that chemokines are very stringently regulated by inflammatory stimuli and are minimally expressed under

Chemokines and CNS Inflammation (cont.)

conditions of normal development or healthy adulthood. Exceptions include chemokines that exert developmental functions, such as stromal cell-derived factor-1 α (SDF-1 α) and are constitutively expressed [29].

Experiments using knockout animals have also shown that chemokines possess functions beyond inflammation. The observation that mice lacking CXCR4 receptors suffer abnormal nervous system development is the first unequivocal demonstration that chemokine receptors transduce essential signals for neural function [30]. Mice deficient for this receptor die *in utero* and exhibit defects in cerebellar development, suggesting that CXCR4 receptor engagement is essential for proper neuronal cell migration and patterning. These data also support an essential role for CXCR4 receptors in hematopoiesis and cardiac development. Cumulatively, these studies clearly show the importance of chemokine and chemokine receptor knockout mice in understanding the roles of chemokines *in vivo*.

Characteristics of inflammation in the mammalian nervous system

Unlike other tissues, the nervous system inflammatory reaction is often restricted to mononuclear phagocytes [31]. For example, macrophages and microglia are major responders to necrosis of CNS neurons following excitotoxic injury induced by systemic injection of kainic acid [32]. Direct mechanical trauma to the cortex or spinal cord also elicits inflammation that is biased heavily towards monocytes and macrophages, after a transient early accumulation of neutrophils [33]. Similarly,

peripheral nervous system Wallerian degeneration, which is the reaction of the distal axonal segment after transection of a nerve, evokes an inflammatory infiltrate that is more than 75% monocytic [34,35]. Each of these insults elicits a unique set of signals to the circulating vascular inflammatory elements, but the cellular responses are relatively uniform. However, the regulatory apparatus that governs this selective recruitment of leukocytes in the nervous system is unknown.

Classical studies by Bell *et al.* demonstrated that the expression of adhesion molecules on microvessels in the CNS is readily induced by injection of lipopolysaccharide (LPS), implying that failure of neutrophil infiltration could not be accounted for by absence of the required adhesive vascular substrate [36]. It has been proposed that neutrophils might fail to enter the CNS after LPS injection (and, by extension, other pathological states) because neutrophil chemoattractants are not elicited by these stimuli. At least two studies favor this notion. Transgenic mice expressing a neutrophil-directed chemokine, growth-related gene product α (GRO α), governed by the CNS-specific myelin basic protein (MBP) promoter, developed massive neutrophil inflammation in the CNS [37]. Similarly, adenoviral expression of a related α -chemokine, macrophage inflammatory protein-2 (MIP-2), in mice resulted in the recruitment of polymorphonuclear leukocytes to brain parenchyma [38]. These results strongly support the hypothesis that the CNS fails to recruit neutrophils because the appropriate chemokines are poorly expressed.

However, selective accumulation of monocytes to the nervous system remains

unexplained. In some animal models, it has been shown that the chemoattractant profile observed in response to CNS trauma is highly restricted to monocyte chemoattractants (primarily MCP-1) at time points that featured monocytic inflammation [39-41]. This result suggests that monocyte-directed chemokines might play a critical role in the restricted infiltration of monocytes into tissue sites following nervous system trauma.

Involvement of chemokines in CNS inflammatory responses

Much work over the past decade has culminated in a scheme, termed the three (or more) signal paradigm, to explain leukocyte extravasation during both physiological and pathological processes. This model is based on sequential and overlapping interactions among several classes of receptors and ligands: first, the selectins and their carbohydrate ligands; second, chemokines and chemokine receptors; and finally, leukointegrins and Ig-superfamily adhesion molecules. There is abundant evidence that this concept has relevance in CNS inflammation [43]. It is currently thought that activated autoimmune T-cells initiate immune-mediated CNS injury by recruitment and activation of inflammatory cells from the bloodstream and from microglia from the CNS. The specific roles of chemokines in this process are now coming into focus. The use of passive immunization with neutralized antibodies has shown that, in the initial phase of EAE, MIP-1 α is required for the inflammatory response. However, relapses of EAE appear to involve MCP-1 [44,45].

Chemokine expression also plays a role in the CNS host response to pathogens, as documented in bacterial and viral models of

meningoencephalitis [46,47]. Moreover, chemokines are selectively upregulated in the cerebrospinal fluid (CSF) of patients with bacterial and viral meningitis, with a direct correlation between the chemotactic properties of the CSF and chemokine levels [48-50]. Most importantly from a public health point of view, there is a strong relationship between CSF MCP-1 levels and HIV dementia, which is of considerable interest given the importance of monocyte recruitment and activation in the CNS during this devastating process [51].

Post-traumatic CNS inflammation may also be governed by local chemokine expression [39]. We have reported selective upregulation of MCP-1 expression by astrocytes after a focal stab-injury to the cerebral cortex of adult mice and also showed upregulation of multiple chemokines in rats that received spinal cord contusion injury [41,52]. Cortical cryoprobe lesioning of

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Chemokines and CNS Inflammation (cont.)

adult mice upregulated MCP-1 both in ipsilateral and contralateral cortex, consistent with a role for MCP-1 in the trans-synaptic signaling that follows neuronal injury [53]. Compatible results have been obtained in studies of cerebral trauma in Lewis rats and several groups have reported increased levels of both α - and β -chemokines following either focal or global cerebral ischemia [54-56]. Selective upregulation of MCP-1 after sterile cerebral trauma was recently shown to correlate with recruitment of macrophages and microglia to the lesions [57].

As noted above, there is direct experimental evidence to support the concept that MIP-1 α and MCP-1 are important for inflammatory cell invasion of the CNS in acute and relapsing EAE [44]. However, chemokines appear to play a more complex role in the regulation of immune-mediated processes than simple chemoattraction of effectors. As one example, the dichotomous process of T-cell commitment to Th1 or Th2 functional differentiation is directly influenced by chemokines: MIP-1 α biases T-cells to Th1 behavior upon antigen challenge while MCP-1 treatment regulates Th2 commitment [58].

Finally, the potential role of chemokines in the pathogenesis of multiple sclerosis (MS) is of interest. One group reported very modest, but elevated MIP-1 α levels in the CSF of MS patients compared to controls, with MIP-1 α concentrations correlating with CSF leukocyte counts [59]. In addition, two MS patients with homozygous absence of functional CCR5 chemokine receptors were recently reported [60]. This finding was not unexpected given recent descriptions of MS pathology that indicate pathogenetic heterogeneity in the disease.

Importantly, recent studies utilizing MS autopsy material confirm cellular sources of chemokines that were initially demonstrated in EAE. Simpson *et al.* showed that the predominant producer of MCP-1 in MS brain is the reactive astrocyte, a finding also demonstrated by others using material from patients that died of HIV dementia [61]. Finally, Hvas *et al.* have shown that RANTES (regulated upon activation normal T expressed and secreted) mRNA is expressed by perivascular lymphocytes in MS brain lesions, as has been previously shown in EAE [62]. In contrast, control brains showed little or no RANTES mRNA expression. These findings may provide avenues for more definitive studies concerning the role of chemokines in MS.

Conclusions

Chemokines play a number of important roles in inflammatory processes, acting as chemoattractants and activators of various inflammatory cells, most notably, leukocytes. However, a variety of findings now challenge such a limited view of chemokine function. A growing body of evidence suggests that, in addition to modulating the recruitment of inflammatory cells responsible for the repair of non-neuronal tissues, chemokines are instrumental in the initiation of repair processes following brain injury. Chemokines also elicit such diverse cellular responses as influencing developmental organogenesis, wound healing, angiogenesis and neoplasia in non-hematopoietic cells. Future studies involving knockout and transgenic models, as well as other animal models and *in vitro* studies, will provide further insight into the specificity and functions of chemokines in the CNS and diseases in which they are involved.

New RBI Product!

L-733,060 HCl

A non-peptide NK₁ substance P receptor antagonist that blocks stress-induced behavioral responses in guinea-pigs

In a landmark paper recently published by scientists from Merck Research Laboratories [1], researchers described the effects of a novel, non-peptide NK₁ substance P receptor antagonist on stress-induced behavioral responses in guinea-pig pups. In initial experiments, data were reported which indicated that pretreatment of guinea-pig pups with the antidepressant drugs **Imipramine** (Cat. No. I-111) or **Fluoxetine** (Cat. No. F-132), or the anti-anxiety drugs **Diazepam** (Cat. No. D-120) or **Buspirone** (Cat. No. B-119), completely blocked an audible vocalization response induced by a period of separation from their mothers and littermates.

Most interestingly, this behavioral response was also blocked by pretreatment with L-733,060, a NK₁ substance P receptor antagonist. These animal studies demonstrated that antagonism of central substance P receptors can inhibit stress-induced behavioral responses in a manner resembling the effects of commonly prescribed psychiatric drugs. In a subsequent clinical trial, also described in the above paper, Merck scientists reported robust antidepressant effects for another NK₁ substance P receptor antagonist, MK-869, in a placebo-controlled trial in patients with severe to moderate depression.

Taken together, these findings suggest not only that **substance P** (Cat. No. S-136) may play an important role in psychiatric disorders, but that blockade of central substance P receptors may represent an entirely new target for antidepressant drug research, an area that has hitherto focused on inhibition of norepinephrine and/or serotonin reuptake.

To aid researchers interested in pursuing these exciting new findings, RBI is pleased to be the first company to offer **L-733,060 HCl** (Cat. No. L-137), a non-peptide, brain penetrating NK₁ substance P receptor antagonist.

1. Kramer, M.S., Cutler, N., Feighner, J. *et al. Science* **281**, 1640-1645 (1998).

L-733,060 is manufactured and sold under license from Merck, Sharp and Dohme Ltd.

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g-Vinyl-GABA - a novel strategy for the treatment of cocaine addiction

In a recent study, Dewey *et al.* [1] reported that γ -vinyl-GABA, a selective and irreversible inhibitor of the enzyme GABA-transaminase, significantly attenuated cocaine-induced increases in neostriatal synaptic dopamine in the non-human primate (baboon) brain as assessed by positron emission tomography (PET). Furthermore, this treatment abolished both the expression and acquisition of cocaine-induced conditioned place preference (CPP), but had no effect on CPP for a food reward, the delivery of cocaine to the brain or locomotor activity. These data suggest that modulation of brain GABAergic mechanisms, using an irreversible inhibitor that raises endogenous GABA levels, might represent an important new therapeutic approach to the treatment of cocaine addiction. For further studies in this important research area, RBI is pleased to offer not only (\pm)- γ -vinyl GABA (Cat. No. V-110), but also its active and inactive enantiomers, **S(+)- γ -vinyl GABA** (Cat. No. V-113) and **R(-)- γ -vinyl GABA** (Cat. No. V-114), respectively.

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Inactive enantiomer of (RS)-AMPA (Cat. No. A-136).
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 *Selective kainate receptor agonist.*
- B-171 1-BCP**
 *Centrally acting AMPA receptor modulator; crosses the blood brain barrier.*
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 *Potent group II metabotropic glutamate receptor agonist.*
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- N-215** **Nocistatin**
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SIGNAL TRANSDUCTION


Adenylyl/Guanylyl Cyclase Products

- I-159** **Isoliquiritigenin**
 (2',4,4'-Trihydroxychalcone)
Soluble guanylyl cyclase activator and aldose reductase inhibitor.
- N-211** **NS 2028**
 Specific soluble guanylyl cyclase inhibitor.

Chemotactic Products

- F-140** **F-Met-Leu-Phe**
 (N-Formyl-Met-Leu-Phe; FMLP)
synthetic; >97% purity Chemotactic peptide; stimulates several cytoplasmic events leading to chemotaxis in neutrophils.

Kinase Related Products

- G-153** **Geldanamycin**
 (GDM)
Blocks the activities of signaling proteins requiring interaction with Hsp90 for proper functioning.

Complete information for all products highlighted in this issue may be obtained at the RBI website: www.callrbi.com. Click on the *Neurotransmissions* icon on our homepage.

New Products from RBI

SIGNAL TRANSDUCTION (cont.)

Kinase Related Products - cont.

P-255 Piceatannol



(3,3',4,5'-Tetrahydroxy-trans-stilbene)
Inhibits the non-receptor tyrosine kinases, Syk and Lck; preferentially inhibits Syk over Lck.

R-126 Radicol



(R2146)
Inhibits Ras-MAP kinase pathway by selectively depleting cells of Raf; inhibits Src tyrosine kinase activity.

U-120 U0126



Succinonitrile; Butanedinitrile
Specific inhibitor of MEK1 and MEK2 (MAP kinase kinase; MAPKK); also inhibits constitutively active, mutant form of MEK.

Nitric Oxide Products

M-230 S-Methyl-L-thiocitrulline acetate



Potent inhibitor of NOS; more potent than L-thiocitrulline (Cat. No. T-173).

Protein Kinase/Phosphatase Related Products

C-272 Cantharidin



(Cantharidine)
Inhibitor of phosphatases 1 and 2A.

K-114 Kemptide



synthetic; >97% purity
Peptide substrate for protein kinase A (cAMP-dependent protein kinase).

Chemokines from RBI



RBI now offers an extensive line of Chemokines and anti-Chemokine antibodies: 48 products, including human, mouse, and rat recombinant chemokines as well as anti-human chemokine antibodies (See page 6). All products are provided lyophilized and are accompanied by a detailed Product Data Sheet.

Antibodies are validated for Western Blot, ELISA, Immunohistochemistry and Neutralization techniques.

Chemokines are validated for use in cell culture assays. Contact the RBI Technical Service Department or your RBI Distributor for additional information.

Receptor Modulating Agent

P-253 Phenylarsine oxide



(PAO; Arzene)
Blocks internalization of cell surface receptors; metabolic poison.

Sphingolipid Signaling Pathway Products

F-142 Fumonisin B1



Inhibitor of sphingosine-N-acetyltransferase.

S-196 Sphingomyelinase



(Sphingomyelin phosphodiesterase)
Initiates the formation of sphingomyelin-based second messengers.

S-195 Sphingosine-1-phosphate



(D-erythro-Sphingosine-1-phosphate)
EDG-1 receptor ligand; involved in cellular signaling; putative lipid second messenger.

Miscellaneous Signaling Products

L-140 IPA



(L- α -Lysophosphatidic acid)
Endogenous agonist for the LPA receptor; putative ligand for EDG-2 and EDG-4.

M-273 Minocycline hydrochloride



(Minocin hydrochloride)
Basement membrane protease inhibitor; inhibits endothelial cell proliferation and angiogenesis.

O-122 Oleamide



(Oleic acid amide)
Sleep inducing brain lipid which allosterically modulates GABA_A receptors and potentiates serotonin receptor responses.

New Antibodies from RBI



RBI now offers an expanded line of antibodies to meet the needs of Neuroscience researchers. New specificities include antibodies to Glutamate Receptors, Neuronal Enzymes, Neuropeptides and Protein Kinases for Signal Transduction studies (See page 17 for details).

Antibodies can be used in a variety of techniques including immunohistochemistry, immunoblotting, ELISA, immunoprecipitation and RIA. Contact the RBI Technical Service Department or your RBI Distributor for additional information including detailed Product Data Sheets.

<i>New Antibodies from RBI</i>		
Cat. No.	Neuropeptides	Size
E-162	Anti- β -Endorphin	200 μ l
E-163	Monoclonal anti-Endothelin, clone ET-1/58	200 μ l
E-164	Anti-Endothelin	200 μ l
E-165	Anti-Leu-enkephalin	500 tests
Signal Transduction Agents		
F-136	Anti-Focal adhesion kinase	200 μ l
J-103	Anti-c-Jun N-terminal kinase	200 μ l
M-267	Anti-MAP kinase kinase	200 μ l
M-268	Anti-MAP kinase kinase 4	200 μ l
M-269	Anti-p38 MAP kinase	200 μ l
P-235	Anti-Phosphatidylinositol 3-kinase	200 μ l
P-236	Anti-Protein kinase C- β_1	200 μ l
P-237	Monoclonal anti-Protein kinase C- β_1 , clone PK-B13	200 μ l
P-238	Anti-Protein kinase C- β_2	200 μ l
P-239	Monoclonal anti-Protein kinase C- β_2 , clone PK-B26	200 μ l
P-240	Anti-Protein kinase C- γ	200 μ l
P-241	Monoclonal anti-Protein kinase C, clone PK-G4	200 μ l
P-242	Anti-Protein kinase C- δ	200 μ l
P-243	Anti-Protein kinase C- ϵ	200 μ l
P-244	Anti-Protein kinase C- ζ	200 μ l
P-245	Anti-Protein kinase C- η	200 μ l
R-127	Anti-Rsk1	200 μ l
S-194	Anti-S6 kinase	200 μ l
Synaptic Proteins		
S-193	Anti-Synapsin I	10 μ g
Glutamate Receptors		
M-264	Anti-N-Methyl-D-aspartate R2A glutamate receptor (NMDA R2A)	10 μ g
M-265	Anti-N-Methyl-D-aspartate R2B glutamate receptor (NMDA R2B)	10 μ g
M-266	Anti-N-Methyl-D-aspartate R2C glutamate receptor (NMDA R2C)	10 μ g
Neuronal Enzymes		
D-216	Anti-Dopamine β -hydroxylase (C-terminal)	30 μ g
D-217	Anti-Dopamine β -hydroxylase (N-terminal)	40 μ g

Application Note

The Use of Quantitative Western Blot Analysis to Determine Expression of NMDA Receptor Subunits

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and Kristin Nixon

University of Colorado
Health Sciences Center
Denver, CO 80262

About the Authors

Michael Browning received his B.A. in English from the University of Texas at Austin in 1970 and his Ph.D. in Biology from the University of California at Irvine. His thesis work with Dr. Gary Lynch involved biochemical studies of long-term potentiation, a putative cellular substrate of memory. His postdoctoral work with Paul Greengard at Yale and Rockefeller University involved immunochemical and biochemical studies of the phosphoprotein synapsin I. Dr. Browning is currently an Associate Professor in the Pharmacology Department of the University of Colorado Health Sciences Center. His work continues to focus on the biochemical basis of long-term potentiation.

Kristin Nixon received her B.S. in Molecular, Cellular and Developmental Biology from the University of Colorado at Boulder in 1993. She currently works as a professional research associate with Dr. Browning where she studies the expression of amino acid receptors in rat brain.

Western blot analysis of proteins using monoclonal and polyclonal antibodies is a routine procedure employed in many laboratories throughout the world [1]. Since its introduction, the technique has undergone much fine-tuning to simplify its use and maximize its effectiveness [2-4]. Although other forms of immunoassay exist, such as ELISA (Enzyme-Linked Immunosorbent Assay) and RIA (Radioimmunoassay), a feature of western blot analysis that makes this form of assay superior is that it allows researchers to identify the molecular weight of the immunoreactive species. Because there is no way to determine the molecular weight of the immunoreactive species when using ELISA or RIA, the identity of the immunoreactive species cannot be ascertained with certainty. Thus, the possibility of obtaining false positive results due to the presence of cross-reactive substances in the assay mixture exists when using ELISA or RIA.

In contrast, western blots can be used to analyze a specific protein even when using antibodies that exhibit cross reactivity. For example, an antibody that recognizes a 100 kDa protein but also cross reacts with a 50 kDa protein, can be used to study the 100 kDa protein in western blots because the immunoreactive signal for the 100 kDa band and the cross reactive band at 50 kDa are easily resolved on an SDS-polyacrylamide gel.

However, this antibody could not be used to reliably assay the 100 kDa protein in an ELISA or RIA, due to the inability of these assays to distinguish the molecular weight of reacting proteins.

Although western blotting has traditionally not been used for quantitative protein analysis, it is becoming more widely used for this purpose. We have used western blot analyses to determine the amounts of synapsin, the synaptic vesicle protein, in microdissected subregions of the rat olfactory bulb [5], in intraocular hippocampal transplants [6,7] and in human postmortem brain tissue from schizophrenics [8]. More recently, we have used western blot analyses to study the expression of specific subunits of the NMDA (N-Methyl-D-Aspartate) glutamate receptor [9]. However, to use western blots to quantify the amount of a particular protein in a sample, certain important issues need to be addressed.

One of the first factors to be established in a quantitative western analysis and in almost any quantitative protein assay is the dynamic working range (DWR) for the particular method and assay conditions. The DWR is the range in an assay where the immunolabeling slope increases significantly with increasing protein load. In western blots, samples with very low or very high immunoreactive signals are typically outside of the DWR and such samples cannot be reliably quantified in the blot. To reliably determine the amount of a particular protein in an experimental sample, the sample mixture must be loaded in an amount that will exhibit immunolabeling

Figure 1.

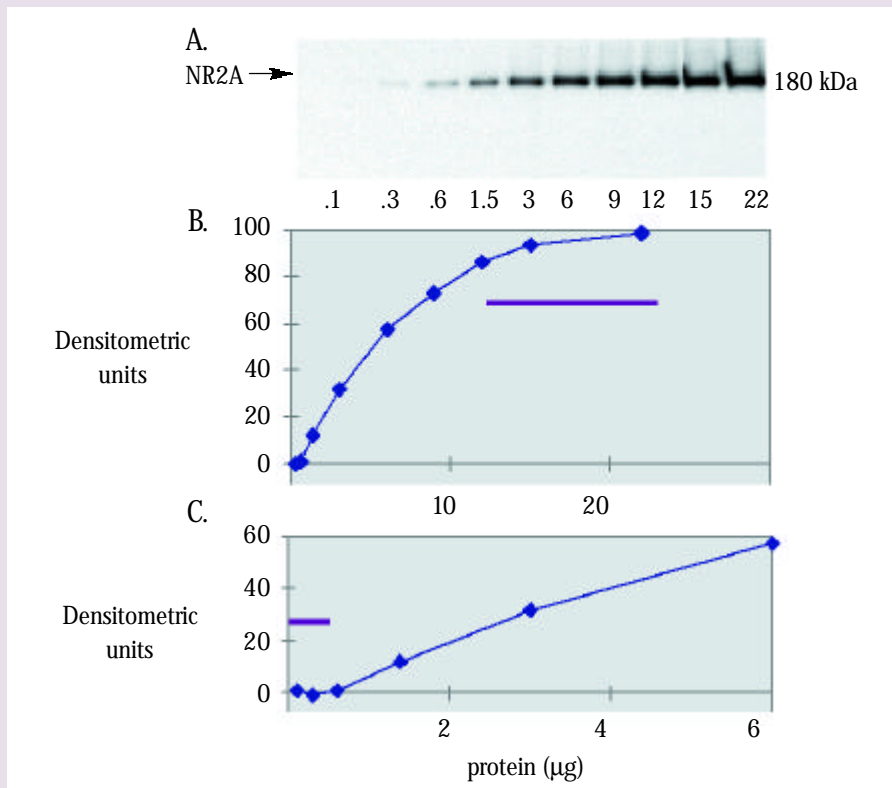
A. ECL exposure from western blot of rat brain homogenate. Samples of homogenate ranging from 0.1 to 22 μg of total protein were loaded on individual lanes of a 7.5% SDS-PAG (sodium dodecyl sulfate-polyacrylamide gel), electrophoresed, then transferred to a PVDF membrane. After blocking in buffer containing 0.05% Tween 20 and 5% non-fat dry milk, the membrane was incubated overnight at room temperature in a 1:2,000 dilution of affinity-purified NMDA receptor NR2A antisera. The membrane was then washed and incubated for 1 hour at room temperature in HRP-conjugated (horse-radish peroxidase-conjugated) goat anti-rabbit IgG and developed with enhanced chemiluminescence (ECL).

B. Graph of immunolabeling of the NR2A subunit as a function of protein loaded.

Exposed film from the developed western blot shown in Figure 1A was subjected to densitometry using the Molecular Analyst program from BioRad. Arbitrary densitometric units for the immunolabeled bands were determined and are plotted versus total protein loaded per lane (in μg). The black bar indicates the upper limit of the DWR.

C. Graph of immunolabeling of the NR2A subunit as a function of protein loaded.

Arbitrary densitometric units for the immunolabeled bands from lanes loaded with 0.1 to 6 μg of total protein were determined as in Figure 1B. Results are plotted versus total protein loaded per lane (in μg). The black bar indicates the lower limit of the DWR.



Application Note (cont.)

within the DWR of the blot. To illustrate this point, a typical western blot using an antibody specific for the NR2A subunit of the NMDA glutamate receptor is shown in Figure 1A. In this blot, increasing amounts of rat brain homogenate (0.1-22 μg) were resolved on the gel and transferred to a PVDF (polyvinylidene fluoride) membrane. The blot was then probed with NR2A affinity-purified antisera (Cat. No. M-264) at a 1:2,000 dilution (the optimal dilution for a particular antibody must be empirically determined and may vary depending on assay conditions and methods of detection) and developed with enhanced chemiluminescence (ECL). Visual inspection of the exposed film suggests that the increase in immunolabeling observed is directly proportional to the amount of protein loaded. However, when immunolabeling is examined by densitometric analysis of the exposed film it is apparent that when high levels of protein have been loaded on the gel the signal saturates. This can be seen in Figure 2B. The graph shows the amount of immunolabeling, represented in arbitrary densitometric units, seen when 0.1 to 22 μg of protein have been loaded in each lane. The immunolabeling signal begins to plateau when greater than 12 μg of protein have been loaded per lane or when immunolabeling signals are greater than 80 densitometric units. The fact that the immunolabeling signal increases very slightly or not at all at high protein concentrations indicates that, in this range, there is not a simple quantitative relationship between the amount of protein loaded and the immunolabeling signal. Therefore, quantification in this region is unreliable. Thus, any sample that gives a response in this range (the region above the solid black line in Figure 1B) is outside the DWR of the assay. In order to accurately determine the amount of specific protein present in such a sample, a smaller amount of

the sample must be run on another blot such that the immunolabeling signal does fall within the DWR of the blot.

Similar concerns exist when attempting to quantify specific proteins within samples of low protein concentration and are illustrated in Figure 1C. The graph shows the amount of immunolabeling for total protein concentrations from 0.1 to 6 μg per lane. The immunolabeling signal does not yield a quantifiable value below 1 μg per lane. Although the developed film yields a visually detectable band at these low protein loads, the signal is outside the DWR. Any sample that gives a response outside of the DWR (the region below the solid black line in Figure 1C) cannot be reliably quantified in this blot. To determine the amount of specific protein in such a sample, a larger amount of the sample must be run on another blot so that its signal does fall within the DWR of the blot.

Once the DWR is determined for a particular antibody and immunolabeling protocol, it is then quite straightforward to begin to use the assay to quantify the relative amounts of a particular protein in the experimental samples of interest. The standard curve is run on the same gel as the samples of interest, and a quantitation of the levels of a particular sample protein can be made by interpolating the signal from the sample within the DWR of the standard curve. It should be emphasized, however, that variables relating to transfer and blot incubation conditions require that a standard curve for determination of the DWR be run for every set of conditions. Any change in the conditions requires a new determination of the DWR.

In summary, western blotting can be a very valuable tool for the quantitative analysis of protein expression. However, the accuracy

New Antibodies to NMDA Receptors, Synapsin and Dopamine β -Hydroxylase from RBI



of this analysis is greatly dependent on the recognition by the experimenter of the quantitative issues described above. It is also important to note that other variables, such as transfer efficiency and membrane capacity which have not been discussed here, will also influence the reliability of the western blot assay for quantitative analyses of protein expression.

References and Notes

1. While the terms 'western blot' and 'dot blot immunoassay', as well as other types of immunoassays, are commonly referred to in the literature under the general term 'immunoblotting', western blots allow separations based on molecular weight while other immunoassays do not. Therefore, the term 'western blot' is most appropriate for this article.
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M-264



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M-265



Anti-NMDA R2B glutamate receptor. Purified rabbit polyclonal antibody for western blot, ELISA, immunohistochemistry, or immunoprecipitation studies in HEK293 cells and rat, mouse and human tissue.

M-266



Anti-NMDA R2C glutamate receptor. Purified rabbit polyclonal antibody for western blot, ELISA, immunohistochemistry, or immunoprecipitation studies in HEK293 cells and rat, mouse and human tissue.

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Anti-Dopamine β -Hydroxylase (C-terminal). Purified sheep polyclonal antibody for blot immunolabeling in human, monkey and bovine tissue and immunohistochemical studies in human and monkey.

D-217



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Tech FAQ's

Frequently Asked Questions received by RBI's Technical Service Group

Q. How is chemokine activity assayed?

A. Chemokines bind to specific G protein-coupled, cell surface receptors on target cells and induce cell type-specific responses, such as cell migration (chemotaxis) or the release of enzyme granules (myeloperoxidase, for example). Therefore, the activity of chemokines towards leukocytes may be measured by cell migration assays or enzyme-based assays.

Cell Migration Assay

In a migration assay, the ability of a specific chemokine to induce leukocyte migration through a porous membrane is measured [1,2]. Leukocytes are plated in the upper well of a double-chambered well. The chemokine is placed in the culture medium of the lower chamber. Upon incubation, the cells migrate through the membrane and subsequently attach to the surface of the membrane exposed to the chemokine. The membrane is fixed and stained, and the number or density of cells that have adhered to the membrane are measured. Thus, the potency of the chemokine towards specific leukocyte subpopulations can be quantitated by plotting cells migrated versus the concentration of the chemokine.

Enzyme-Based Assay

Since chemokines may also stimulate the release of enzyme granules, a second way to measure chemokine activity is by measuring enzyme activity in the medium of chemokinetreated cells. For example, stimulated neutrophils release myeloperoxidase [3]. In the presence of myeloperoxidase, the chemical o-phenylenediamine dihydrochloride (OPD) is oxidized, resulting in a solution that absorbs light and can be quantitated colorimetrically. As with migration assays, the amount of enzyme activity is directly proportional to the chemokine concentration.

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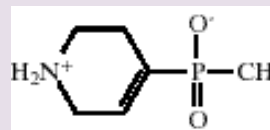
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TPMPA: Selective GABA_C receptor antagonist

GABA_C receptors are defined by their insensitivity to the GABA_A receptor antagonist **bicuculline** (Cat. No. G-003) and the GABA_B receptor agonist **baclofen** (Cat. No. B-020). **TPMPA** [(Cat. No. T-200); (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid], is the first highly selective (K_b 2.1 μM) GABA_C receptor antagonist and exhibits little affinity for GABA_A (K_b 320 μM) or GABA_B (EC₅₀ 500 μM) receptors. Although there is increasing evidence to indicate that GABA_C receptors are present in various brain regions, the currently available information on GABA_C receptors comes from studies performed in the visual system, particularly in the retina. **TPMPA** provides a useful tool for

studying the function of the GABA_C receptors and will aid in identifying GABA_C receptor-mediated responses in other parts of the CNS as well. **TPMPA** was first synthesized by Dr. Ricardo Miledi at the University of California, Irvine and is sold by RBI in the USA under an exclusive license by the University of California.

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Neurotransmissions Volume 14, No. 2, October 1998
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Editor: Keith J. Watling, Ph.D.
Associate Editor: Susan G. Macdonald, Ph.D.
Graphics/Production: David S. Maltais

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