ELSEVIER

Journal of Chemical Neuroanatomy 17 (1999) 45–55

Journal of CHEMICAL NEUROANATOMY

www.elsevier.com/locate/jchemneu

Localization of the cGMP-dependent protein kinases in relation to nitric oxide synthase in the brain

A.E.-D. El-Husseini [a], J. Williams [a], P.B. Reiner [a], S. Pelech [b], S.R. Vincent [a,*]

[a] *Graduate Program in Neuroscience, Department of Psychiatry, Division of Neurological Sciences, The University of British Columbia, Vancouver, Canada BC V6T 1Z3*

[b] *Department of Medicine, The University of British Columbia, Vancouver, Canada BC V6T 1Z3*

Received 17 February 1999; received in revised form 8 June 1999; accepted 27 June 1999

Abstract

The distributions of the type I and type II isoforms of cGMP-dependent protein kinase were determined in the rat brain using immunohistochemistry and in situ hybridization, and compared with the localization of NO synthase determined with NADPH-diaphorase histochemistry. The type I cGMP-dependent protein kinase was highly expressed in the Purkinje cells of the cerebellar cortex, where it was closely associated with the NO synthase containing granule and basket cells. This kinase was also found in neurons in the dorsomedial nucleus of the hypothalamus, where it may be regulated by NO or atriopeptides. The type I kinase was not detected in other central neurons. In contrast, the type II kinase was widely distributed in the brain. In particular, it was highly expressed in the olfactory bulb, cortex, septum, thalamus, tectum and various brainstem nuclei. Many regions expressing this kinase also contained, or received innervation from NO synthase positive neurons. These results indicate that type I cGMP-dependent protein kinase may act as a downstream effector for NO only in the cerebellar cortex and the dorsomedial hypothalamus. The type II cGMP-dependent protein kinase appears to be a major mediator of NO actions in the brain.

Keywords: Cyclic GMP; Nitric oxide; Protein kinase; Immunohistochemistry; In situ hybridization

1. Introduction

Cyclic guanosine 5′-monophosphate (cGMP) mediates the physiological actions of two distinct intercellular messengers in the nervous system; the atriopeptides and nitric oxide (NO). Atriopeptides act on a unique family of cell surface receptors, the particulate guanylyl cyclases, which have a single transmembrane domain and an intracellular catalytic domain (Garbers, 1992). NO, which can diffuse freely across cell membranes, acts on the soluble isoforms of guanylyl cyclase, binding to the heme moiety of the protein, which causes its activation (Ignarro, 1991; Hobbs, 1997). The resultant increases in cGMP are thought to underlie the physiological actions of the atriopeptides and NO.

There appear to be three main classes of intracellular targets for cGMP, cGMP-regulated phosphodiesterases, cGMP-gated ion channels and cGMP-dependent protein kinases. By altering phosphodiesterase activity, cGMP can regulate the intracellular levels of cAMP (Beavo, 1995). cGMP-gated ion channels are well studied in photoreceptors and olfactory epithelium, and more recent studies have shown that they are also expressed in the brain (El-Husseini et al., 1995a). Finally, by activating specific protein kinases, cGMP may regulate the function of numerous substrate proteins to affect cell function in a manner analogous to that of cAMP acting through cAMP-dependent protein kinase.

Two distinct types of cGMP-dependent protein kinase have been identified in the brain, which differ in their catalytic and regulatory properties (Gamm et al., 1995). Type I (cGKI) occurs in two isoforms which arise from alternative splicing of a single gene which is highly expressed in smooth muscle, lung and platelets (Sandberg et al., 1989; Wernet et al., 1989). Biochemi-

* Corresponding author. Tel.: + 1-604-822-7038; fax: + 1-604-822-7981.

E-mail address: svincent@unixg.ubc.ca (S.R. Vincent)

0891-0618/99/$ - see front matter
PII: S0891-0618(99)00023-X

cal and immunohistochemical studies indicate that in the brain cGKI is concentrated in the Purkinje cells in the cerebellum (Lohmann et al., 1981; De Camilli et al., 1984).

A second gene, encoding a distinct cGMP-dependent protein kinase (cGKII) was recently characterized from mouse brain and rat intestine (Uhler, 1993; Jarchau et al., 1994). More recently, others have cloned the human cGKII, and mapped it to chromosome 4 (Fujii et al., 1995; Orstavik et al., 1996). The various cAMP-dependent protein kinase isoforms are differentially expressed in the brain, indicating that functional differences in cAMP responses may be mediated by specific kinases (Cadd and McKnight, 1989). Likewise we showed using reverse transcriptase-PCR analysis and in situ hybridization that the distribution of cGKII is much different from that of cGKI, with cGKII like NO synthase, being widely expressed in the brain (El-Husseini et al., 1995b). This led us to advance the hypothesis that cGKII is a major target for NO/cGMP signalling in the nervous system. In the present report we have determined the distribution of the two cGMP-dependent protein kinases in the brain in detail. We also compare the relationship of NO synthase-positive neurons to those expressing the cGMP-dependent protein kinases.

2. Materials and methods

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. Adult male Wistar rats were obtained from the Animal Care Centre of the University of British Columbia. They were deeply anaesthestized with pentobarbital and perfused through the ascending aorta with 100 ml 0.9% NaCl, followed by 300 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brain was dissected out, immersed in fixative for 2 h at 4°C, and then incubated in 15% sucrose in 0.1 M phosphate buffered saline (PBS) overnight at 4°C. Coronal and parasaggital sections were cut at 30 μm on a freezing microtome and collected in PBS containing 0.02% sodium azide for immunohistochemistry. For in situ hybridization, 10 μm thick sections were thaw-mounted onto 3-aminopropyltriethoxysilane/chrome alum-coated slides and stored at −80°C. All solutions were prepared with RNAse-free reagents and diethyl pyrocarbonate-treated, autoclaved water.

2.1. Immunohistochemistry and enzyme histochemistry

A 16 residue synthetic peptide (CDEPPPDDNSGWDIDF) corresponding to the conserved C-terminal of bovine and human cGKIα and β was synthesised and coupled to keyhole limpet hemocyanin, and used to immunize New Zealand white rabbits. The sera was applied onto an agarose column to which the immunogen was thio-linked. Antibody was eluted from the column with 0.1 M glycine (pH 2.5) and the antibody solution was neutralized to pH 7.0 with saturated Tris base.

The antibody was tested using Western blotting. Homogenates from rat cerebellum, cortex, thalamus and pons-medulla were separated on 8.5% SDS-PAGE gels, and transferred to nitrocellulose membranes which were incubated in blocking solution of 50 mM Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 (TBST) and 5% milk powder. After rinsing, the membranes were incubated with primary antibody overnight at 4°C in TBST containing 1% bovine serum albumin (BSA). The membranes were then rinsed and incubated with horseradish peroxidase-linked secondary antibodies (1:5000 dilution; Amersham). After washing, immunoreactive proteins were detected with enhanced chemilumenescence (ECL, Amersham).

For immunohistochemistry the ABC immunoperoxidase method was used as previously described (Vincent et al., 1994). Brain sections were obtained from male Sprague–Dawley rats perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were rinsed in 15% sucrose and 30 μm thick sections were cut on a freezing microtome and washed in 0.02 M PBS. The antibody to cGKI was diluted 1:5000 in PBS containing 0.3% Triton X-100 (PBST) and 2% normal goat serum (NGS) for 24 h at 4°C. The sections were then rinsed 3×20 min in PBST, and incubated with biotinylated sheep anti-rabbit secondary antibody (Vector Laboratories) diluted 1:1000 in PBST containing 2% NGS for 1 h at room temperature. The sections were again rinsed 3×20 min in PBST, and incubated for 1 h with an avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Laboratories, Burlingame, CA) in PBST. Following a final series of rinses in PBST, the immunoreactivity was revealed using diaminobenzidine with nickel ammonium sulphate (Vincent et al., 1994). Control sections were processed in parallel with the exception that normal rabbit serum was used in place of the cGKI antibody. Also, sections and Western blots were examined using antibody preabsorbed overnight with the synthetic peptide antigen (50 μg/ml antibody, diluted 1:10), and these showed no immunoreactivity.

The expression of cGKI and NO synthase was examined on single sections using a double staining procedure (Vincent et al., 1994). The sections were first stained for NADPH-diaphorase histochemically to localize NO synthase (Hope et al., 1991). The sections were incubated in PBS containing 1 mg/ml β-NADPH and 0.1 mg/ml nitro blue tetrazolium, with 0.3% Triton X-100, at 37°C for 30–60 min. They were then rinsed

thoroughly in PBS and stained for cGKI-immunoreactivity using the method described above. This method resulted in blue staining of NO synthase containing cells and processes, and brown staining of cGKI-immunoreactive structures, allowing the simultaneous detailed examination of the dendritic and axonal processes of both populations of stained cells. Sections incubated for NADPH-diaphorase without NADPH served as a control.

2.2. In situ hybridization

The frozen sections were thawed in 4% paraformaldehyde, incubated with 1 μg/ml proteinase K for 10 min, and then treated with 0.25% acetic anhydride–1.5% triethanolamine. The sections were sequentially dehydrated in 50, 70, 90, and 95% ethanol, then rehydrated. Sense and antisense ^{35}S-labelled riboprobes were synthesized using T7 and Sp6 RNA polymerases, respectively, from a 713-bp cGK II PCR product subcloned into the PCRII vector (El-Husseini et al., 1995b). Probes were prepared in a hybridization solution containing 600 mM NaCl, 10 mM Tris (pH 7.5), 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone, 1 mM EDTA, 0.01% salmon sperm DNA, 10% dextran, 0.1% sodium dodecyl sulphate, 0.1% sodium thiosulphate, 100 mM dithiothreitol, and 0.005% yeast tRNA. The probe was applied at 5×10^5 cpm to the sections on a slide, and incubated for 16 h at 54°C. Following hybridization the sections were rinsed with 2 × saline–sodium citrate (SSC), treated with RNAse (20 μg/ml) for 30 min, washed with 2 × SSC at 55°C for 1 h, and then with 0.1 × SSC at 60°C for 2 h. The slides were then exposed to X-Omat X-ray film (Kodak) for 2–3 weeks.

3. Results

3.1. Type I cGMP-dependent protein kinase

The affinity-purified antibody to cGK1 was first characterized using Western blotting. Crude protein extracts were prepared from rat cerebellum, cortex, thalamus and pons-medulla, and resolved by SDS-PAGE. After blotting, the membrane was incubated with the antibody to cGKI and detected with chemiluminescence. A single band of about 76 kDa was detected at antibody dilutions of 1:5000 in the cerebellar extracts but not in other brain regions (Fig. 1).

We next undertook immunohistochemical studies to determine in detail the distribution of cGKI in the brain. Consistent with the Western blotting, and previous immunohistochemical studies (Lohmann et al., 1981; De Camilli et al., 1984), the Purkinje cells of the cerebellar cortex were found to be intensely stained for cGKI (Fig. 2A). The staining was found throughout the dendritic tree in the molecular layer, and in the cell soma. The Purkinje cell nuclei were unstained. The axons leaving the Purkinje cells through the granule cell layer, and into the subcortical white matter were also well stained. The nerve terminals of the Purkinje cells in the deep cerebellar nuclei, and in the vestibular nucleus were also well stained.

The rest of the brain was thoroughly examined for cGKI immunoreactivity, however, only one other brain region displayed positive staining. A small cluster of neurons in the compact portion of the dorsomedial nucleus of the hypothalamus displayed positive cGKI immunoreactivity (Fig. 2B and C). Staining was evident in cell bodies and short dendritic processes in this region (Fig. 3A and B). Staining of the dorsomedial hypothalamic neurons and the Purkinje cells was not seen when the antibody was preabsorbed with the peptide antigen prior to immunostaining.

Double staining for NADPH-diaphorase and cGKI was consistent with previous reports that in the cerebellar cortex, NO is produced by NO synthase in granule cells and their processes and in the basket cells in the molecular layer, but not in the Purkinje cells, which were negative for NADPH-diaphorase activity. NO produced in these compartments would, therefore, be in close proximity to the cGKI in the Purkinje cell bodies and dendrites (Fig. 3D and E).

In the hypothalamus, double staining also indicated that the cGKI-positive cells did not express NO synthase, although there were weakly-stained NO synthase neurons within the dorsomedial nucleus. At the caudal levels, the cGKI-positive cell group in this area was

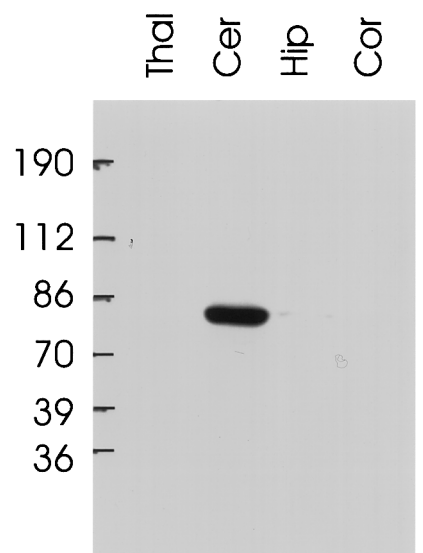

Fig. 1. Western blot of rat brain homogenates detected using affinity-purified rabbit antibody to cGKI. The 76 kDa kinase was highly expressed in the cerebellum (Cer), but not in the thalamus (Thal), hippocampus (Hip) or cortex (Cor).

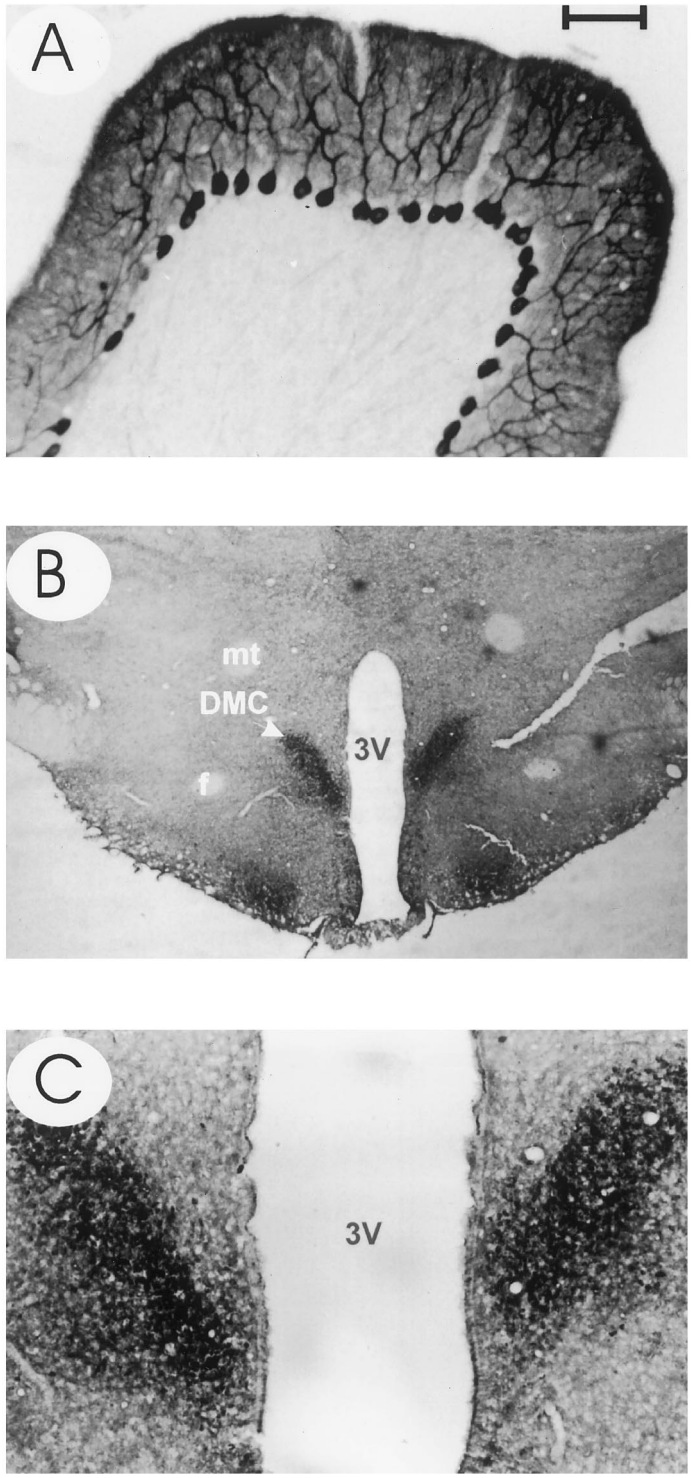

Fig. 2. Immunohistochemical localization of cGKI in the rat brain. (A) The dendrites, cell bodies and axons of the Purkinje cells of the cerebellar cortex are intensely stained. (B, C) Many, closely clustered, small cells of the compact portion of the dorsomedial nucleus of the hypothalamus also displayed staining for the kinase. The scale bar indicates 100 μm in A, 500 μm in B and 130 μm in C.

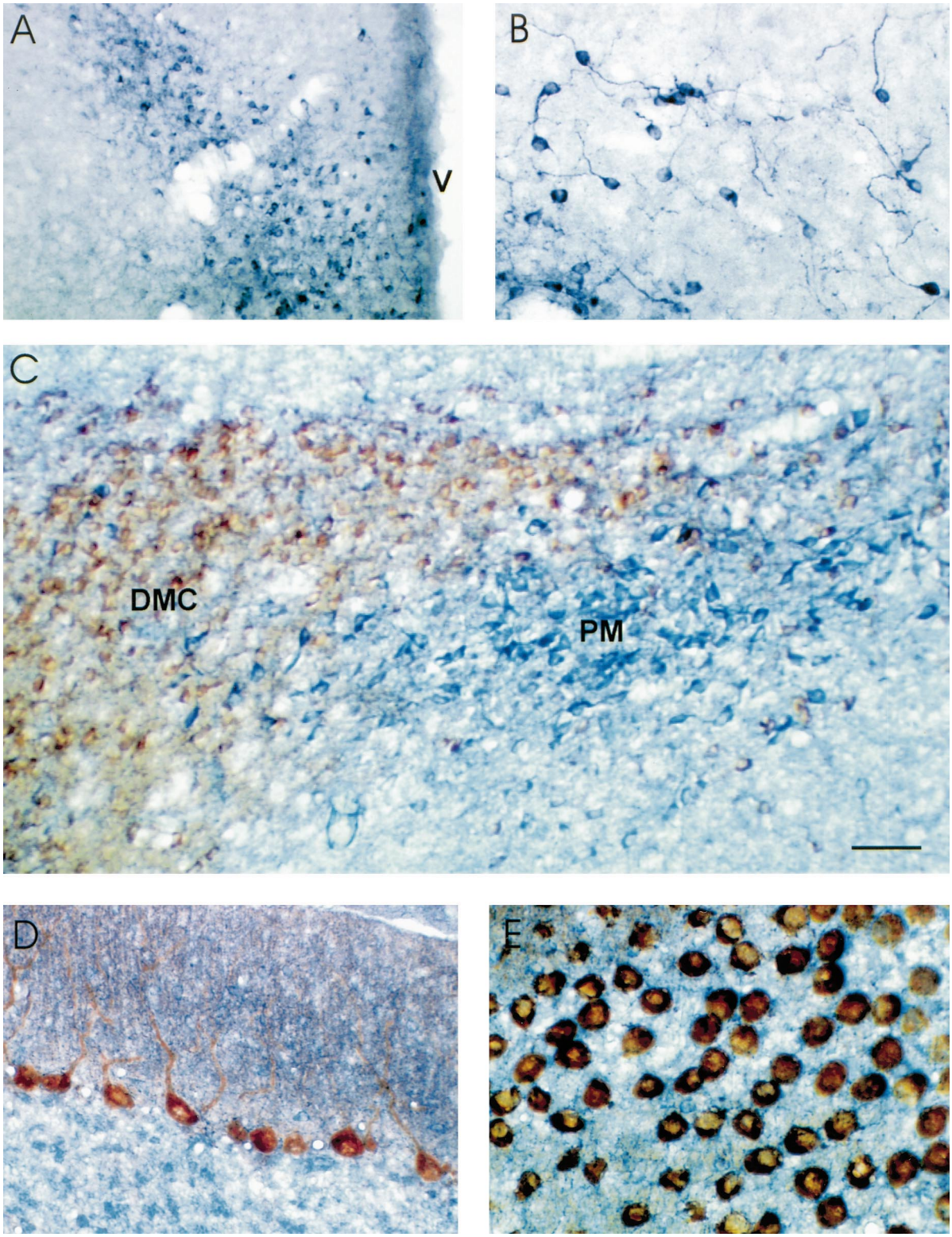

Fig. 3. Immunoperoxidase staining of cGKI in the dorsomedial hypothalamic nucleus, illustrates the cluster of positive neurons along the third ventricle (V) (A). At higher magnification the unstained nuclei of these small cells are apparent, as are their many stained processes (B). (C–E) Double staining for NADPH-diaphorase histochemistry with a blue formazan reaction, and immunoperoxidase staining for type I cGMP-dependent protein with a brown DAB reaction. In the hypothalamus (C) the kinase-positive cells of the dorsomedial nucleus (DMC) do not express NO synthase, but are closely associated with NADPH-diaphorase positive cells in the premammillary (PM) nucleus. In the cerebellar cortex, NO synthase is present in granule cells and basket cells, while the kinase is concentrated in the Purkinje cells (D). In a tangential section through the Purkinje cell layer, the kinase-positive Purkinje cell bodies can be seen to be surrounded by stained basket cells and their processes (E). Scale bar indicates 250 μm in A, and 100 μm in B, C, D and E.

closely associated with NO synthase positive neurons in the perifornical, dorsal premammillary and supramammillary nuclei (Fig. 3C).

3.2. Type II cGMP-dependent protein kinase

In contrast to cGKI, which had a very restricted distribution in the brain, cGKII was widely expressed. In situ hybridization demonstrated cGKII mRNA in neurons throughout the brain. High levels of cGKII mRNA were obvious in the olfactory bulb, the outer layers of the cerebral cortex, the septum, the thalamus, the superior colliculus, the locus ceruleus, the pontine nuclei and the nucleus of the solitary tract (Fig. 4). Other regions showing high levels of cGKII expression included the pyriform cortex, and the lateral amygdala (Fig. 5).

The extensive expression of cGKII in the thalamus was analyzed in further detail in a series of coronal sections (Fig. 5). In the anterior thalamus dense labelling was observed in the paratenial nucleus, the anterior medial and the anteroventral nuclei (Fig. 5A). The laterodorsal nucleus showed strong labelling, while the ventral nuclei were less intensely labelled (Fig. 5B). In the midline the intralaminar nuclei were also labelled with the reuniens nucleus displaying particularly high labelling. The medial habenula was also intensely labelled (Fig. 5B and C). At more caudal levels, there was pronounced labelling in the lateral geniculate nucleus, the intralaminar nuclei and the posterior thalamic nuclei (Fig. 5C and D). The subthalamic nucleus was also very strongly labelled (Fig. 5C and D).

4. Discussion

Although the distribution of NO synthase in the brain is well known, and the role of the NO/cGMP signal transduction system is beginning to be understood, little is known regarding the localization of the downstream effectors in this pathway. A key mediator of signalling via NO and cGMP appears to be the cGMP-dependent protein kinase. In the vascular system, activation of cGKI by the NO/cGMP pathway is thought to underlie endothelium-dependent relaxation (Butt et al., 1993). Two isoforms of cGKI have been identified which arise from alternative splicing from a single gene. The type Iα and type Iβ isoforms differ only in the N-terminal 100 amino acids or so, the rest of the molecules being identical (Butt et al., 1993). Both isoforms are expressed in vascular smooth muscle, while type Iα is the predominant form in the lung and the cerebellum (Keilbach et al., 1992).

We have generated an antibody to the conserved C-terminal region of cGKI, which should detect both the α and the β isoforms. Western blotting demonstrated that the antibody could detect a single protein band of 76 kDa in the cerebellum, corresponding to the predicted size of cGKIα (Sandberg et al., 1989; Wernet et al., 1989;

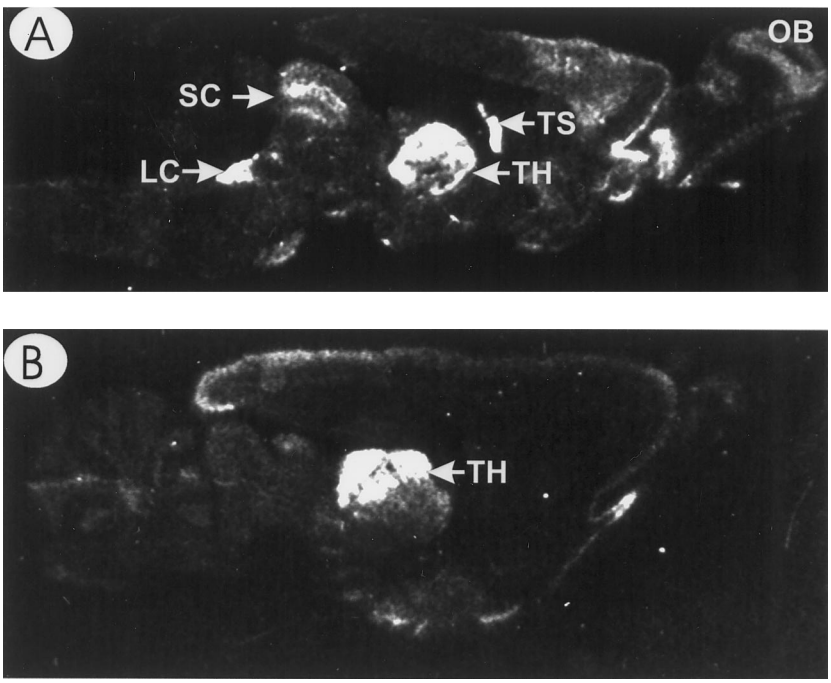

Fig. 4. Expression of cGKII mRNA in the rat brain determined with in situ hybridization. Detection of kinase mRNA in parasaggital sections was achieved with an antisense riboprobe generated from the kinase cDNA. Very strong labelling was detected in the thalamus (TH), the triangular septum (TS) and locus ceruleus (LC). Strong labelling was seen in the superior colliculus (SC), olfactory bulb (OB) and the outer layers of the neocortex. Other brain regions had low or undetectable levels of expression.

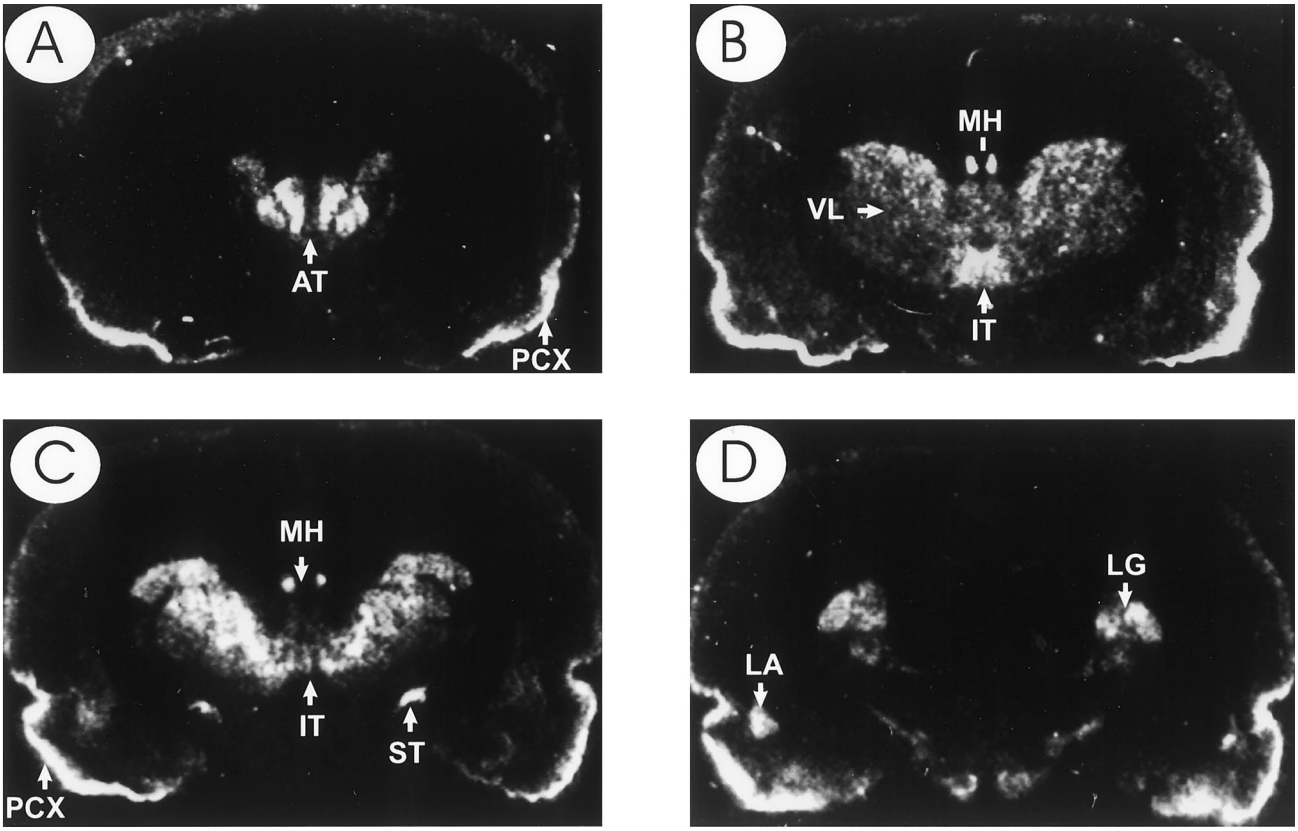

Fig. 5. Expression of cGKII in darkfield images of coronal sections through the rostral (A) to caudal (D) extent of the rat thalamus. At rostral levels the kinase was highly expressed in the paratenial nucleus and the anteriomedial nucleus of the anterior thalamus (AT). Very high expression was also detected in the medial habenula (MH), subthalamic nucleus (ST), lateral amygdala (LA), lateral geniculate (LG) and pyriform cortex (PCX). The intralaminar nuclei of the thalamus (IT) express high levels of cGKII, while the ventrolateral nucleus (VL) expresses moderate levels.

Butt et al., 1993). Immunohistochemical analysis indicated that cGKI was very highly expressed in the Purkinje cells, being present within the dendritic trees, cell bodies, axons and nerve terminals. These observations are consistent with previous work using other antibodies (Lohmann et al., 1981; De Camilli et al., 1984).

The NADPH-diaphorase histochemistry indicates that in the cerebellar cortex NO synthase is present in the granule cells and their parallel fibres and in basket cells in the molecular layer, as noted previously using this method or NO synthase immunohistochemistry (Vincent, 1996). A major downstream target of the NO produced in these compartments would appear to be the cGKI that is concentrated in the Purkinje cell bodies and dendrites. Evidence from in situ hybridization and immunohistochemistry indicates that soluble guanylyl cyclase is also highly expressed in Purkinje cells (Ariano et al., 1982; Nakane et al., 1983; Matsuoka et al., 1992; Furuyama et al., 1993).

Although the axons and nerve terminals of the Purkinje cells express high levels of cGKI immunoreactivity, they do not appear to innervate NO synthase positive neurons. The neurons of the deep cerebellar nuclei and the vestibular nucleus do not express NO synthase, or NADPH-diaphorase activity (Vincent and Kimura, 1992; Rodrigo et al., 1994). Thus the role of cGKI in the Purkinje cell axons and terminals in these areas is puzzling.

We have discovered that the cells of the dorsomedial nucleus of the hypothalamus express cGKI immunoreactivity. Previous studies have demonstrated that neurons in this nucleus express high levels of galanin immunoreactivity (Skofitsch and Jacobowitz, 1985; Melander et al., 1986). The dorsomedial nucleus of the hypothalamus is thought to be involved in reproductive, cardiovascular, locomotor, circadian and ingestive behaviours (Bernardis and Bellinger, 1987; Luiten et al., 1987). It may also play a role in hypothalamic responses to stress. These actions appear to be mediated by the extensive connections between the dorsomedial nucleus and other nuclei of the hypothalamus (Ter Horst and Luiten, 1986; Luiten et al., 1987; Thompson et al., 1996). The supraoptic and paraventricular nuclei express NO synthase and innervate the dorsomedial nucleus. This suggests that NO-induced cGMP in the dorsomedial nucleus may play a role in the regulation of fluid balance via the subfornical organ and its reciprical connections with these regions. Indeed, lesions of the dorsomedial nucleus induces hypodipsia (Bernardis and Bellinger, 1987).

The cGKI immunoreactive cells in the hypothalamus may also respond to atriopeptides following the activation of particulate guanylyl cyclases. de Vente et al. (1989) have demonstrated strong cGMP immunostaining in neurons in what appears to be the dorsomedial hypothalamic nucleus (c.f. their Fig. 16) after stimulation with atrial natriuritic peptide. Indeed, these appear to be the only neurons in the brain which show an increase in cGMP in response to the atriopeptide (de Vente et al., 1989). NPR-B is the primary natriuretic peptide receptor in the hypothalamus, and by inference CNP would be predicted to be the primary active natriuretic peptide in this region (Langub et al., 1995).

In marked contrast to the restricted distribution of cGKI, cGKII is very widely expressed throughout the brain. In the cortex, cGKII mRNA, like that for soluble guanylyl cyclase (Matsuoka et al., 1992; Furuyama et al., 1993; Burgunder and Cheung, 1994) is concentrated in layers II–III, and is particularly high in the pyriform cortex. These regions contain a dense network of NO synthase fibres derived from local neurons present throughout the cortex (Vincent and Kimura, 1992; Rodrigo et al., 1994). Fibre networks in the outer layers of the cerebral cortex also display a dense staining for NO-induced cGMP-immunoreactivity (de Vente et al., 1998).

We could detect no cGKI staining in the hippocampal formation using our antibodies which are specific for the C-terminal of cGKI and do not recognize cGKII. Furthermore, cGKII mRNA expression was very low in the hippocampus. Likewise, we could not detect any cGKI immunoreactivity or cGKII expression in the striatum. This contrasts to one earlier report in which staining of medium-spiny cells was noted (Ariano, 1983). It is interesting to note that regions such as the hippocampus and striatum, which express high levels of soluble guanylyl cyclase (Matsuoka et al., 1992; Furuyama et al., 1993; Burgunder and Cheung, 1994), but do not appear to express much if any cGMP-dependent protein kinase, do express very high levels of a cGMP-stimulated phosphodiesterase (Sonnenburg et al., 1991; Repaske et al., 1993). Thus, in these regions the major role of cGMP may be to regulate cAMP levels via changes in phosphodiesterase activity, rather than to directly activate a protein kinase. Indeed there is evidence that the actions of cGMP on calcium currents in hippocampal neurons are mediated through phosphodiesterase-induced reductions in cAMP levels (Doerner and Alger, 1988).

The thalamus expresses very high levels of cGKII mRNA. Thalamic expression of soluble guanylyl cyclase has also been described (Furuyama et al., 1993; Burgunder and Cheung, 1994). In the adult thalamus, NO synthase-containing neurons are restricted to the paraventricular, rhomboid and central medial nuclei of the midline, and the dorsal and ventral lateral geniculate (Mitrofanis, 1992; Vincent and Kimura, 1992; Gabbott and Bacon, 1994; Bertini and Bentivoglio, 1997). High densities of NADPH-diaphorase positive fibres are found in the anteroventral and anteromedial nuclei, the midline nuclei, the anterior intralaminar nuclei and the geniculate (Vincent and Kimura, 1992; Bertini and Bentivoglio, 1997). The fibres in the anteroventral and anteromedial nuclei may arise from the NO synthase-positive neurons of the medial mammillary nucleus, the laterodorsal and pedunculopontine tegmental nuclei or the ventral lateral geniculate (Vincent and Kimura, 1992; Bertini and Bentivoglio, 1997). The mesopontine NO synthase neurons and hypothalamic NO synthase neurons innervate the midline nuclei (Otake et al., 1995). Indeed, we have shown that stimulation of the mesopontine tegmental neurons leads to an increase in NO production in the thalamus (Williams et al., 1997) and a NO-dependent activation of cGKII (El-Husseini et al., 1998).

The subthalamic nucleus is one regions in which cells appear to express both NO synthase (Vincent and Kimura, 1992; Endoh et al., 1994; Northington et al., 1996) and cGKII. Subthalamic neurons also express high levels of soluble guanylyl cyclase (Giuili et al., 1994). Indeed, both NOS-immunoreactivity and NO-induced cGMP-immunoreactivity have been detected in subthalamic neurons and fibres (de Vente et al., 1998). This indicates that NO produced in subthalamic neurons could act in an autocrine fashion, activating cGKII in these cells. NO synthase itself may be one potential target for cGKII phosphorylation (Dinerman et al., 1994).

It is puzzling that the medial habenula is almost devoid of any NADPH-diaphorase or NO synthase activity, while the lateral habenula has an intense network of NO synthase fibres and terminals (Vincent and Kimura, 1992; Bertini and Bentivoglio, 1997). In contrast, the medial habenula expresses among the highest levels of mRNA for soluble guanylyl cyclase and cGKII in the brain (Matsuoka et al., 1992; Furuyama et al., 1993; Burgunder and Cheung, 1994; El-Husseini et al., 1995b). It may well be that the soluble guanylyl cyclase and cGKII are transported to the terminals of the medial habenular neurons in the interpeduncular nucleus, a region containing a large number of NO synthase neurons (Vincent and Kimura, 1992; Rodrigo et al., 1994). A dense network of NO-induced cGMP staining is seen in the interpeduncular nucleus (de Vente et al., 1998). Thus NO produced by neurons in the interpeduncular nucleus may serve to increase cGMP in the axon terminals of the medial habenular neurons.

The locus ceruleus expresses very high levels of soluble guanylyl cyclase (Matsuoka et al., 1992; Furuyama et al., 1993). We have found high expression of cGKII in this region as well. These observations are consistent with electrophysiological studies indicating that NO can affect locus ceruleus activity via cGMP-dependent protein kinase (Xu et al., 1994; Pineda et al., 1996). The locus ceruleus neurons do not express NO synthase, and the

source of endogenous NO which could affect these neurons remains to be determined. The laterodorsal and pedunculopontine tegmental nuclei lie just medial to the locus ceruleus and express very high levels of NO synthase (Vincent and Kimura, 1992) and soluble guanylyl cylclase (Furuyama et al., 1993). Indeed, in both there is strong labelling of fibres throughout this regions for NO-induced cGMP immunoreactivity (de Vente et al., 1998). Thus NO may diffuse from these cells to act on the locus ceruleus neurons.

The targets for cGMP-dependent phosphorylation in the brain are largely unknown. We have demonstrated that cGKII undergoes cGMP-dependent phosphorylation in neurons, and this can be regulated in vivo by NO (El-Husseini et al., 1998). In addition, we observed a number of other thalamic proteins whose phosphorylation was regulated by NO and cGMP, however, these remain to be characterized (El-Husseini et al., 1998). Others have recently noted that many substrate proteins for cGMP-dependent protein kinase are present in brain (Wang and Robinson, 1995, 1997).

The best characterized neuronal substrate for cGKI is G-substrate, a small protein with some similarities to the protein phosphatase inhibitors DARPP-32 and inhibitor 1 (Aswad and Greengard, 1981). G-substrate co-localizes with cGKI in the Purkinje cells of the cerebellar cortex (Detre et al., 1984). It has also been reported to be present in the neuropil of the thalamus (Qian et al., 1996), suggesting that it may also function as a substrate for cGKII, which we have shown to be highly expressed in thalamic neurons. G-substrate has recently been cloned (Hall et al., 1999), and in situ hybridization by this group indicates specific expression in the Purkinje cells and what appears to be the dorsomedial hypothalamic nucleus (c.f. their Fig. 4C). These observations indicate that in both of these two cell groups cGKI and G-substrate may form a specific signal transduction pathway.

The phosphorylation of protein phosphatase inhibitor 1 in smooth muscle by cGKI has been demonstrated (Tokui et al., 1996), indicating that this may be one mechanism by which cGMP can modify the calcium-sensitivity of these cells. Furthermore, both NO and the atriopeptides have been shown to result in cGMP-dependent phosphorylation of DARPP-32 (Tsou et al., 1993). Together these observations indicate that inhibition of protein phosphatases may be an important downstream effect of cGMP. Other potential targets for cGMP-dependent phosphorylation have been identified in some systems. Phosphorylation of ryanodine receptor purified from skeletal muscle (Suko et al., 1993) and IP_3 receptors from smooth muscle (Komalavilas and Lincoln, 1994) by cGKI has been documented. The present results point to cGKII as a major downstream effector for NO action in the brain. It will thus be crucial to identify the neuronal substrates for this protein kinase.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada. S.R.V. is an MRC Senior Scientist, and S.P. and P.B.R. are MRC Scientists. We thank Chris Bladen for technical assistance.

References

Ariano, M.A., Lewicki, J.A., Brandwein, H.J., Murad, F., 1982. Immunohistochemical localization of guanylate cyclase within neurons of rat brain. Proc. Natl. Acad. Sci. USA 79, 1316–1320.

Ariano, M.A., 1983. Distribution of components of the guanosine 3′,5′-phosphate system in rat caudate-putamen. Neuroscience 10, 707–723.

Aswad, D.W., Greengard, P., 1981. A specific substrate from rabbit cerebellum for guanosine 3′,5′-monophosphate-dependent protein kinase. I. Purification and characterization. J. Biol. Chem. 256, 3487–3493.

Beavo, J.A., 1995. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. 75, 725–748.

Bernardis, L.L., Bellinger, L.L., 1987. The dorsomedial hypothalamic nucleus revisited, 1986 update. Brain Res. 434, 321–381.

Bertini, G., Bentivoglio, M., 1997. Nitric oxide synthase in the adult and developing thalamus-histochemical and immunohisochemical study in the rat. J. Comp. Neurol. 388, 89–105.

Burgunder, J.M., Cheung, P.T., 1994. Expression of soluble guanylyl cyclase gene in adult rat brain. Eur. J. Neurosci. 6, 211–217.

Butt, E., Geiger, J., Jarchau, T., Lohmann, S.M., Walter, U., 1993. The cGMP-dependent protein kinase, gene, protein and function. Neurochem. Res. 18, 27–42.

Cadd, G., McKnight, G.S., 1989. Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain. Neuron 3, 71–79.

De Camilli, P., Miller, P.E., Levitt, P., Walter, U., Greengard, P., 1984. Anatomy of cerebellar Purkinje cells in the rat determined by a specific immunohistochemical marker. Neuroscience 11, 761–817.

Detre, J.A., Nairn, A.C., Aswad, D.W., Greengard, P., 1984. Localization in mammalian brain of G-substrate, a specific substrate for guanosine 3′,5′-cyclic monophosphate-dependent protein kinase. J. Neurosci. 4, 2843–2849.

de Vente, J., Bol, J.G.J.M., Steinbusch, H.W.M., 1989. cGMP-producing, atrial natriuretic factor-responding cells in the rat brain, an immunocytochemical study. Eur. J. Neurosci. 1, 436–460.

de Vente, J., Hopkins, D.A., Markerink-Van Ittersum, M., Emson, P.C., Schmidt, H.H.H.W., Steinbusch, H.W.M., 1998. Distribution of nitric oxide synthase and nitric oxide-receptive, cyclic GMP-producing structures in the rat brain. Neuroscience 87, 207–241.

Dinerman, J.L., Steiner, J.P., Dawson, T.M., Dawson, V., Snyder, S.H., 1994. Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. Neuropharmacology 33, 1245–1251.

Doerner, D., Alger, B.E., 1988. Cyclic GMP depresses hippocampal Ca^{2+} current through a mechanism independent of cGMP-dependent protein kinase. Neuron 1, 693–699.

El-Husseini, A.E.-D., Bladen, C., Vincent, S.R., 1995a. Expression of the olfactory cyclic nucleotide gated channel (CNG1) in the rat brain. NeuroReport 6, 1331–1335.

El-Husseini, A.E.-D., Bladen, C., Vincent, S.R., 1995b. Molecular characterization of a type II cyclic GMP-dependent protein kinase expressed in the rat brain. J. Neurochem. 64, 2814–2817.

El-Husseini, A.E.-D., Bladen, C., Williams, J.A., Reiner, P.B., Vincent, S.R., 1998. Nitric oxide regulates cyclic GMP-dependent protein kinase phosphorylation in rat brain. J. Neurochem. 71, 676–683.

Endoh, M., Maiese, K., Wagner, J.A., 1994. Expression of the neural form of nitric oxide synthase by CA1 hippocampal neurons and other central nervous system neurons. Neuroscience 63, 679–689.

Fujii, M., Ogata, T., Takahashi, E., et al., 1995. Expression of the human cGMP-dependent protein kinase II gene is lost upon introduction of SV40 T antigen or immortalization in human cells. FEBS Lett. 375, 263–267.

Furuyama, T., Inagaki, S., Takagi, H., 1993. Localizations of α1 and β1 subunits of soluble guanylate cyclase in the rat brain. Mol. Brain Res. 20, 335–344.

Gabbott, P.L., Bacon, S.J., 1994. Two types of interneuron in the dorsal lateral geniculate nucleus of the rat: a combined NADPH diaphorase histochemical and GABA immunocytochemical study. J. Comp. Neurol. 350, 281–301.

Gamm, D.M., Francis, S.H., Angelotti, T.P., Corbin, J.D., Uhler, M.D., 1995. The type II isoform of cGMP-dependent protein kinase is dimeric and possesses regulatory and catalytic properties distinct from the type I isoforms. J. Biol. Chem. 270, 27380–27388.

Garbers, D.L., 1992. Guanylyl cyclase receptors and their endocrine, paracrine and autocrine ligands. Cell 71, 1–6.

Giuili, G., Luzi, A., Poyard, M., Guellaën, G., 1994. Expression of mouse brain soluble guanylyl cyclase and NO synthase during ontogeny. Dev. Brain Res. 81, 269–283.

Hall, K.U., Collins, S.P., Gamm, D.M., Massa, E., DePaoli-Roach, A.A., Uhler, M.D., 1999. Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate — a Purkinje cell substrate of the cyclic GMP-dependent protein kinase. J. Biol. Chem. 274, 3485–3495.

Hobbs, A.J., 1997. Soluble guanylate cycase: the forgotten sibling. Trends Pharmacol. Sci. 18, 484–491.

Hope, B.T., Michael, G.J., Knigge, K.M., Vincent, S.R., 1991. Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA 88, 2811–2814.

Ignarro, L.J., 1991. Heme-dependent activation of guanylate cyclase by nitric oxide: a novel signal transduction mechanism. Blood Vessel. 28, 67–73.

Jarchau, T., Hausler, C., Markert, T., et al., 1994. Cloning, expression, and in situ localization of rat intestinal cGMP-dependent protein kinase II. Proc. Natl. Acad. Sci. USA 91, 9426–9430.

Keilbach, A., Ruth, P., Hofmann, F., 1992. Detection of cGMP-dependent protein kinase isozymes by specific antibodies. Eur. J. Biochem. 208, 467–473.

Komalavilas, P., Lincoln, T.M., 1994. Phosphorylation of the inositol 1,4,5-triphosphate receptor by cyclic GMP-dependent protein kinase. J. Biol. Chem. 269, 8701–8707.

Langub, M.C. Jr, Dolgas, C.M., Watson, R.E. Jr, Herman, J.P., 1995. The C-type natriuretic peptide receptor is the predominant natriuretic peptide receptor mRNA expressed in rat hypothalamus. J. Neuroendocrinol. 7, 305–309.

Lohmann, S.M., Walter, U., Miller, P.E., Greengard, P., De Camilli, P., 1981. Immunohistochemical localization of cyclic GMP-dependent protein kinase in mammalian brain. Proc. Natl. Acad. Sci. USA 78, 653–657.

Luiten, P.G.M., Ter Horst, G.J., Steffens, A.B., 1987. The hypothalamus, intrinsic connections and outflow pathways to the endocrine system in relation to the control of feeding and metabolism. Prog. Neurobiol. 28, 1–54.

Matsuoka, I., Giuili, G., Poyard, M., et al., 1992. Localization of adenylyl and guanylyl cyclase in rat brain by in situ hybridization: comparison with calmodulin mRNA distribution. J. Neurosci. 12, 3350–3360.

Melander, T., Hökfelt, T., Rökaeus, A., 1986. Distribution of galanin-like immunoreactivity in the rat central nervous system. J. Comp. Neurol. 248, 475–517.

Mitrofanis, J., 1992. NADPH-diaphorase reactivity in the ventral and dorsal lateral geniculate nuclei of rats. Vis. Neurosci. 9, 211–216.

Nakane, M., Ichikawa, M., Deguchi, T., 1983. Light and electron microscopic demonstration of guanylate cyclase in rat brain. Brain Res. 273, 9–15.

Northington, F.J., Koehler, R.C., Traystman, R.J., Martin, L.J., 1996. Nitric oxide synthase 1 and nitric oxide synthase 3 protein expression is regionally and temporally regulated in fetal brain. Dev. Brain Res. 95, 1–14.

Orstavik, S., Solberg, R., Tasken, K., et al., 1996. Molecular cloning, cDNA structure, and chromosomal localization of the human type II cGMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 220, 759–765.

Otake, K., Ruggiero, D.A., Nakamura, Y., 1995. Adrenergic innervation of forebrain neurons that project to the paraventricular thalamic nucleus in the rat. Brain Res. 697, 17–26.

Pineda, J., Kogan, J.H., Aghajanian, G.K., 1996. Nitric oxide and carbon monoxide activate locus coeruleus neurons through a cGMP-dependent protein kinase: involvement of a nonselective cationic channel. J. Neurosci. 16, 1389–1399.

Qian, Y., Chao, D.S., Santillano, D.R., et al., 1996. cGMP-dependent protein kinase in dorsal root ganglion, relationship with nitric oxide synthase and nociceptive neurons. J. Neurosci. 16, 3130–3138.

Repaske, D.R., Corbin, J.G., Conti, M., Goy, M.F., 1993. A cyclic GMP-stimulated cyclic nucleotide phosphodiesterase gene is highly expressed in the limbic system of the rat brain. Neuroscience 56, 673–686.

Rodrigo, J., Springall, D.R., Uttnthal, O., et al., 1994. Localization of nitric oxide synthase in the adult rat brain. Phil. Trans. R. Soc. Lond. B 345, 175–221.

Sandberg, M., Natarajan, V., Ronander, I., et al., 1989. Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-dependent protein kinase from human placenta. FEBS Lett. 255, 321–329.

Skofitsch, G., Jacobowitz, D.M., 1985. Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. Peptides 6, 509–546.

Sonnenburg, W.K., Mullaney, P.J., Beavo, J.A., 1991. Molecular cloning of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase cDNA. J. Biol. Chem. 266, 17655–17661.

Suko, J., Maurer-Fogy, I., Plank, B., et al., 1993. Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-, cGMP- and CaM-dependent protein kinase. Biochim. Biophys. Acta 1175, 193–206.

Ter Horst, G.J., Luiten, P.G., 1986. The projections of the dorsomedial hypothalamic nucleus in the rat. Brain Res. Bull. 16, 231–248.

Thompson, R.H., Canteras, N.S., Swanson, L.W., 1996. Organization of projections from the dorsomedial nucleus of the hypothalamus, a PHA-L study in the rat. J. Comp. Neurol. 376, 143–173.

Tokui, T., Brozovich, F., Ando, S., Ikebe, M., 1996. Enhancement of smooth muscle contraction with protein phosphatase inhibitor 1 — activation of inhibitor 1 by cGMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 220, 777–783.

Tsou, K., Snyder, G.L., Greengard, P., 1993. Nitrix oxide/cGMP pathway stimulates phophorylation of DARPP-32, a dopamine and cAMP-regulated phosphoprotein, in the substantia nigra. Proc. Natl. Acad. Sci. USA 90, 3462–3465.

Uhler, M.D., 1993. Cloning and expression of a novel cyclic GMP-dependent protein kinase from mouse brain. J. Biol. Chem. 268, 13586–13591.

Vincent, S.R., 1996. Nitric oxide and synaptic plasticity: NO news from the cerebellum. Behav. Brain Sci. 19, 362–367.

Vincent, S.R., Kimura, H., 1992. Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46, 755–784.

Vincent, S.R., Das, S., Maines, M.D., 1994. Brain heme oxygenase isoenzymes and nitric oxide synthase are co-localized in select neurons. Neuroscience 63, 223–231.

Wang, X., Robinson, P.J., 1995. Cyclic GMP-dependent protein kinase substrates in rat brain. J. Neurochem. 65, 595–604.

Wang, X., Robinson, P.J., 1997. Cyclic GMP-dependent protein kinase and cellular signalling in the nervous system. J. Neurochem. 68, 443–456.

Wernet, W., Flockerzi, V., Hofmann, F., 1989. The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. FEBS Lett. 251, 191–196.

Williams, J., Vincent, S.R., Reiner, P.B., 1997. Nitric oxide production in rat thalamus changes with behavioral state, local depolarization and brainstem stimulation. J. Neurosci. 17, 420–427.

Xu, Z.Q., Pieribone, V.A., Zhang, X., Gillner, S., Hökfelt, T., 1994. A functional role for nitric oxide in locus coeruleus: immunohistochemical and electrophysiological studies. Exp. Brain Res. 98, 75–83.