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Nitric Oxide and Glutathione as Modulators of Thalamic Sensory Neurotransmission in the Rat

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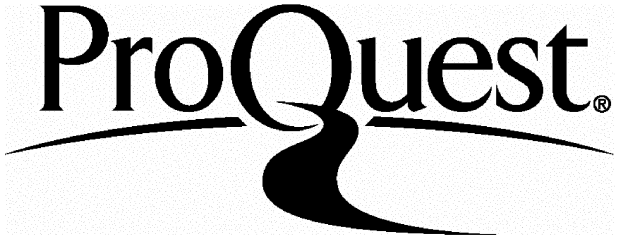

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PUBLICATIONS ARISING FROM DATA INCLUDED IN THIS THESIS

1. The Effects of the Nitric Oxide-Donors Sodium Nitroprusside (SNP) and S-Nitrosoglutathione (GSNO) on Rat Ventrobasal Thalamus Neurones *In Vivo*.

SHAW, P.J. & SALT, T.E. (1996). *British Journal of Pharmacology* **117 (Suppl.)**, 25P.

2. Modulation of Sensory and Excitatory Amino Acid (EAA) Responses by Nitric Oxide (NO) Donors and Glutathione in the Rat Ventrobasal (VB) Thalamus.

SHAW, P.J. & SALT, T.E. (1996). *Society For Neuroscience Abstracts* **22**, 606.12.

3. Modulation of Sensory and Excitatory Amino Acid Responses by Nitric Oxide Donors and Glutathione in the Ventrobasal Thalamus of the Rat.

SHAW, P.J. and SALT, T.E. (1997). *European Journal of Neuroscience* In Press.

4. Sensory and Excitatory Amino Acid Responses in Rat Thalamus *In Vivo* are Potentiated by Iontophoretic Application of 8-Bromo-Cyclic GMP.

SHAW, P.J. & SALT, T.E. (1997). *Brain Research Association Abstracts* **14**,6.01.

5. Potentiation of Sensory Responses by 8-Bromo-Cyclic GMP in Lateral Geniculate Nucleus (LGN) of the Rat Thalamus *In Vivo* and *In Vitro*.

SHAW, P.J., CHARLES, S.L. & SALT, T.E. (1997). *Society For Neuroscience Abstracts* **23**, Submitted.

ABSTRACT

The ventrobasal complex of the thalamus (VB) and the dorsal lateral geniculate nucleus (dLGN) are the thalamic nuclei that carry information regarding somatosensory and visual modalities, respectively. Thalamic sensory nuclei do not, however, passively convey information to the cerebral cortex; rather, they are capable of complex modulations of the pattern of neuronal firing.

The intercellular messenger nitric oxide has a number of postulated roles in synaptic transmission in the CNS. There is accumulating evidence that nitric oxide is present in the thalamus and that it has a role in sensory neurotransmission.

This was designed to investigate the involvement of nitric oxide in neurotransmission in VB and dLGN, using a variety of pharmacological tools. Using an *in vivo* rat preparation, drugs were applied to relay neurones by iontophoresis; by combining this technique with single cell extracellular recording, it was possible to determine the effects of drugs which interact with the nitric oxide system.

The results indicate that nitric oxide donors have a facilitatory action both in VB and dLGN, supporting previous work which suggests that nitric oxide has an excitatory action in the thalamus. Similarly, evidence is presented which supports the suggestion that thalamic nitric oxide exerts its effects via stimulation of guanylate cyclase to increase the intracellular concentration of cGMP.

Additionally, the effects of glutathione were tested using the same experimental protocols; the results indicate that both reduced and oxidised glutathione have an inhibitory action in VB and dLGN.

Given that the thalamic sensory nuclei have an important role in arousal, the

pharmacology of agents which modulate the responsiveness of relay neurones is of great significance. The evidence presented supports the suggestion that nitric oxide has a facilitatory function in the gating of sensory information in the thalamus, and proposes that glutathione has an inhibitory role in the same physiological processes.

CHAPTER 1

INTRODUCTION

I.1. THE THALAMUS

I.1.(i). THE THALAMUS: GENERAL INTRODUCTION

The thalamus is a cluster of related structures located in the diencephalon; it is subdivided into nuclei on the basis of its connectivity and cell morphology, of which the `relay' nuclei of the dorsal thalamus are the most extensively studied. These nuclei receive ascending sensory and motor information and relay it to discrete areas of the cerebral cortex (reviewed by Jones, 1985; Price, 1995). Other parts of the thalamus, the ventral thalamus and the epithalamus, do not have efferent projections to the cerebral cortex and will not be considered in subsequent paragraphs, which will discuss the role of the thalamus as a modulator of information *en route* to the cortex.

The sensory relay nuclei can be identified according to sensory modality. The dorsal lateral geniculate nucleus (dLGN) relays information regarding vision from the retinal ganglion cell layer principally to the primary visual cortex. The ventrobasal complex (VB) relays in a similar manner somatosensory information regarding tactile sensation, joint position and pain to the somatosensory cortex. The medial geniculate nucleus carries auditory information (Jones, 1985).

The thalamus is not, however, simply the location of a relay synapse in ascending pathways. The relay nuclei are under the influence of several factors, including the brainstem (see for example Sillito *et al*, 1983; McCormick and Prince, 1987), the

thalamic reticular nucleus (e.g. Steriade *et al*, 1985) and the cerebral cortex itself (e.g. Wise and Jones, 1977), all of whom may serve to modify or even gate the information ascending to the cortex. It has been proposed with justification that the thalamic sensory relay nuclei are the locations of a complex mechanism to control the flow of information to the cortex and hence to influence the attentiveness or arousal of the animal, involving both extrathalamic neuronal elements and the intrinsic properties of thalamic relay neurones (Jones, 1985).

I.1.(ii). ORGANISATION, ANATOMY AND CIRCUITRY OF THE RAT THALAMUS

I.1.(ii).a. *The ventrobasal complex*

The region of the rat thalamus referred to as the ventrobasal complex (VB) can be divided into two subcompartments, the ventral posterolateral nucleus (VPL) and the ventral posteromedial nucleus (VPM), according to the origin of ascending input. For most purposes, their functions and organisation are identical, hence the use of the single term VB [discussed in a historical perspective by Jones (1985)]; the two subcompartments will be considered separately in subsequent paragraphs only to distinguish their inputs and receptive field properties, and afterwards will be discussed together.

VPL receives somatosensory afferents carrying information about tactile stimulation and joint position from the dorsal column nuclei (the cuneate and gracile nuclei) via the medial lemniscus, and from the spinal cord via the spinothalamic tract. It therefore contains a somatotopic representation of the contralateral trunk, limbs and tail (Angel and Clarke, 1975).

VPM receives somatosensory afferent fibres from trigeminal nuclei. As such, VPM contains a representation of the contralateral face and head, with an especially large proportion of neurones devoted to information regarding the facial vibrissae (whiskers); the rodent makes extensive use of its vibrissae as tactile exploratory devices, hence the large area of VPM given over to their input. As in VPL, neurones in VPM are somatotopically organised, with distortion of the size of the representation to reflect the relative importance of inputs (Waite, 1973a&b). The dorsal vibrissae are represented in a vertical column at the caudal end of VPM; neurones whose receptive fields are the most posterior vibrissae are located at the dorsal end of the columns. Ventral vibrissae are similarly organised more rostrally in VPM. Thus, a vertical microelectrode penetration through the rat VPM from dorsal to ventral encounters neurones whose receptive fields are of an approximately single row, moving from caudal vibrissae to rostral vibrissae with increasing depth (Waite, 1973a). In rodents, VPM is enlarged with respect to VPL, with the converse being true in primates which use the hand and feet as the primary tactile organs.

Receptive fields of VB neurones are typically small and clearly defined areas of the body surface; in VPM, many neurones respond to deflection of a single vibrissa, which adapt to maintained stimulation (Waite, 1973a&b).

Cells in rat VB are morphologically of only one type; unlike the dLGN of the same species and VB and dLGN of the cat, rat VB has few or no inhibitory interneurones (McAllister and Wells, 1981; Ohara *et al*, 1983; Webster and Rowe, 1984). Immunohistochemical studies have confirmed earlier Golgi studies: Harris and Hendrickson (1985) found only a very small number of neurones in rat VB which were positive for glutamic acid decarboxylase (GAD), an immunohistochemical marker for

GABA synthesis. Inhibition in rat VB, therefore, comes exclusively from the thalamic reticular nucleus (TRN), which is discussed below. Also unlike the dLGN (of any species) neurones in the rat VB cannot be morphologically subcategorised (Harris, 1986); in cat dLGN, for instance, neurones can be morphologically (as well as physiologically) distinguished into X, Y and W groups (Guillery, 1966; Friedlander *et al*, 1981).

In addition to ascending sensory input from the principal trigeminal nucleus, the dorsal column nuclei and the spinothalamic tract, VB makes connections with several other sources: these are the thalamic reticular nucleus, the cerebral cortex (which are discussed below) and the brainstem (which is discussed in section I.1.(iv).).

The target for axons of VB neurones is the primary somatosensory cortex (S1), on the ipsilateral side. Terminations are mostly in layer IV of this region, with less dense innervation of layers V and VI (Lu and Lin, 1993). The somatosensory cortex (the upper part of layer VI) also projects back to VB, in a reciprocal manner (Wise and Jones, 1977; Bourassa *et al*, 1995). At least in the cat dLGN, corticothalamic synapses constitute a large proportion of synapses onto relay cells, with each geniculocortical cell receiving input from many corticogeniculate cells [discussed and calculated by Sherman and Koch (1986)].

The thalamic reticular nucleus (TRN) borders the thalamus proper, and can be divided into subregions which connect with individual thalamic nuclei and regions of the cerebral cortex. For example, the TRN contains a region with afferent connections from the dLGN; the cells in this region are visually responsive (and in fact in the cat, this region is given a specific name, the perigeniculate nucleus or PGN). Similarly, the TRN possesses a region whose modality corresponds to its connections with VB and the

somatosensory cortex (Ohara and Lieberman, 1985). The circuitry of the VB/TRN/S1 relationship is such that VB and S1 both project to TRN via collaterals of thalamocortical and corticothalamic fibres, while GABAergic TRN neurones project back to VB (De Biasi *et al*, 1988; Pinault *et al*, 1995). TRN also receives modulatory cholinergic, noradrenergic and serotonergic influence from the brainstem (Steriade *et al*, 1988; Hallanger and Wainer, 1988; Kayama *et al*, 1982; Cropper *et al*, 1984). As the GABAergic nature of the TRN neurones would imply, the function of the TRN is to provide feedback inhibition to VB and the other thalamic relay nuclei (Kayama, 1985; Bal and McCormick, 1993). The role of the TRN may be more sophisticated, however, as has been revealed by the work of Steriade *et al* (1985), who have described the changes which occur in the firing pattern of both thalamocortical cells and TRN cells during the transition from sleep to attentiveness (burst firing to single spike firing, see later) which is thought to be under the control of brainstem modulatory neurones. Steriade *et al* (1985) also report high frequency burst firing in thalamocortical cells which have been disconnected from the TRN, implying a role for the TRN in the control of thalamocortical firing mode.

FIGURE 1: *Neuronal connections of the rat ventrobasal thalamus*

(overleaf)

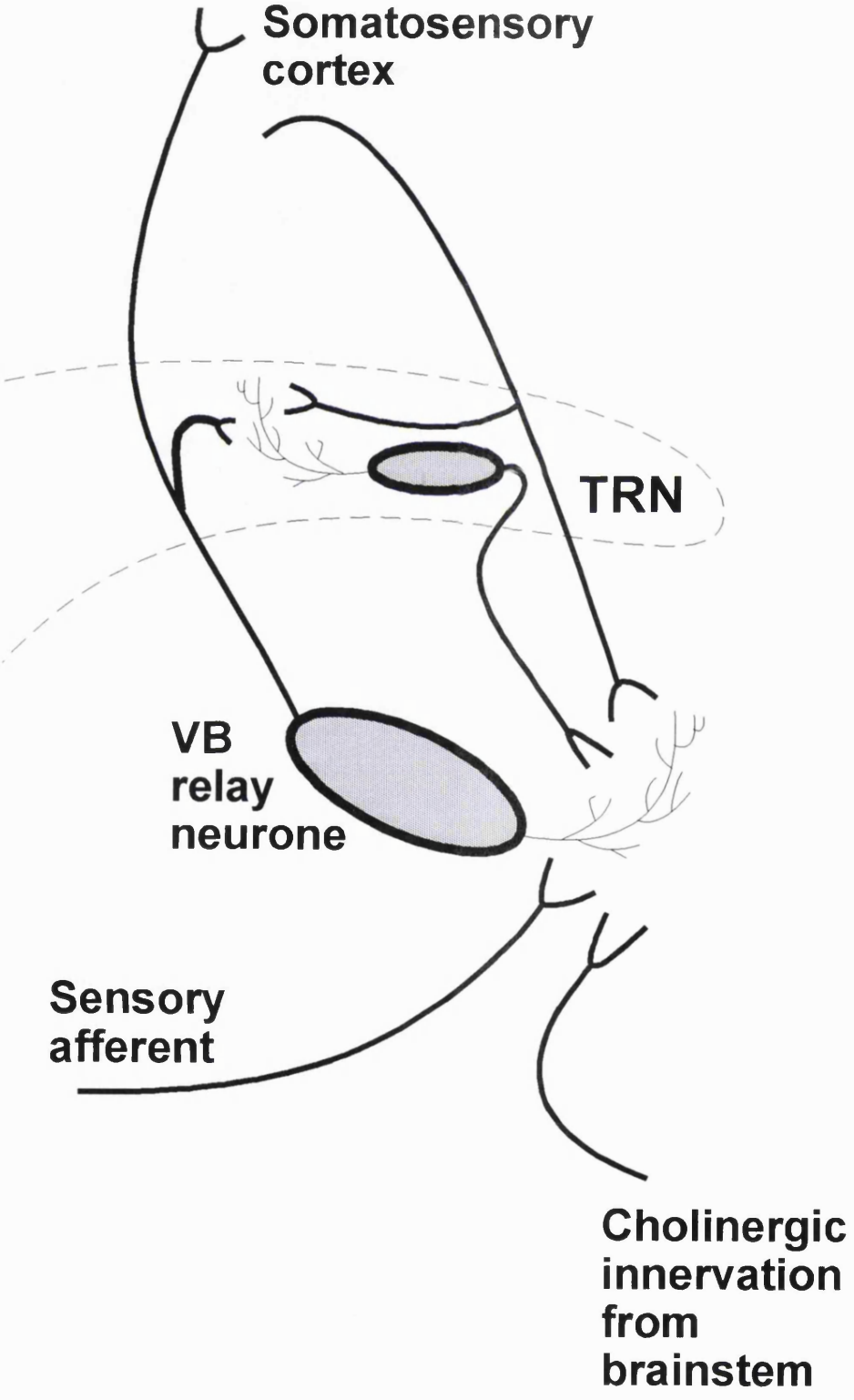

Somatosensory cortex
TRN
VB relay neurone
Sensory afferent
Cholinergic innervation from brainstem

FIGURE 1

Neuronal connections of the rat ventrobasal thalamus

This figure schematically represents the major afferent and efferent neuronal projections and circuitry of the ventrobasal thalamus of the rat. See section I.1.(ii).a.

I.1.(ii).b. *The Dorsal Lateral Geniculate Nucleus of the Rat: Input, Neuronal Morphology and Projections*

It has been generally accepted that the dorsal lateral geniculate nucleus of the rat differs in several key respects from that of the more extensively studied cat and primate. Most significantly, histological examination of the feline or primate dLGN reveals a clearly laminated structure, based upon cytological differences, for instance such as exists between the magnocellular and parvocellular divisions (Guillery, 1970). In the rat, by contrast, microscopic examination of the dLGN indicates a more homogeneous structure, with only subtle morphological variations between cells in different regions of the nucleus. This may be misleading however, as more detailed investigation has shown that the dLGN of the rat does have a regionally distinct anatomy, based upon segregation of retinal and other inputs (Lund *et al*, 1974; Lund and Cunningham, 1972; Reese, 1988).

In the cat and primate, the characteristic laminar organisation of the dLGN is a result of segregated inputs from the ipsilateral and contralateral retinae. Despite the absence of a distinct lamination in the rat, studies in which anterograde neuronal tracers have been injected into one or the other retina have revealed some degree of segregation of crossed and uncrossed retinogeniculate afferents in dLGN. The uncrossed terminations appear to lie in the medial portion of the nucleus, extending some distance rostrocaudally, but absent from the most caudal quarter. Both the crossed and the uncrossed portions of the pigmented rat's dLGN appear to have a topographical organisation, such that the centro-peripheral axis of the visual field is represented mediolaterally, while the superior to inferior visual axis is represented rostrocaudally (Reese and Cowey, 1983; Reese and Jeffrey, 1983; Reese and Cowey, 1987; Reese,

1988).

The retina of the rat consists of three principal classes of morphologically distinct ganglion cells. These classes differ in their soma size (type I (or L) the largest, 5% of the total population, type II (or M) intermediate, 28% of the total, and type III (or S) the smallest, 67% of the total) (Fukuda, 1977), in the extent of their dendritic fields (Perry, 1979) and in the diameters of their axons (Sumitomo *et al* 1969 , Fukuda, 1977). Studies using horseradish peroxidase (HRP) as a tracer have shown that the `inner core' of the dLGN (i.e. the rostroventral portion) is innervated by a small number of ganglion cells primarily of the class I. The outer, caudodorsal portion of the dLGN received afferents from the smaller classes of retinal ganglion cells, with a greater number of these cells being labelled by HRP (Martin, 1986). The same study also showed that dLGN receives input from only 37% of retinal ganglion cells.

The receptive field properties of neurones in the rat dLGN can be characterised on the basis of tonic or phasic responsiveness, spatial summation, etcetera, into groups resembling the W and Y classes of cells in the cat dLGN; the rat possesses very few cells with receptive field properties similar to those of the cat X cells (Fukuda *et al*, 1979; Hale *et al*, 1979).

It can be concluded with some confidence, therefore, that the rat's dLGN is segregated into two major regions. The inner, rostroventral region receives afferents from a relatively greater number of type I retinal ganglion cells, which have rapidly conducting axons and which terminate in small arborisations. Input is from the contralateral side of the visual field (the contralateral nasal and ipsilateral temporal retinae, segregated into sublaminae). The outer, dorsocaudal region principally receives afferents from the smaller, more slowly conducting types II and III retinal ganglion cells,

forming synapses with smaller relay cells. The source of ganglion cell input to the outer shell is solely from the contralateral retina.

Input to the rat dLGN from the superior colliculus (SC) is also confined to a specific area; anterograde tracing studies following injection into the superficial layers of SC reveal connections with the caudodorsal aspect of the ipsilateral dLGN. This projection too has a topographic organisation (Reese, 1984).

Non-visual input to the dLGN comes principally from five brainstem regions. These are the dorsal raphe, the mesencephalic reticular formation, periaqueductal grey matter, dorsal tegmental nucleus and locus coeruleus (Pasquier and Villar, 1982; Mackay-Sim *et al*, 1983). These inputs are bilateral, although the ipsilateral brainstem predominates. Brainstem structures are known to have modulatory influence on thalamic responsiveness (see below).

I.1.(ii).b.1. Cell morphology in rat dLGN

Neurones in dLGN are commonly subdivided into two broad categories: relay cells and interneurones (Burke and Sefton 1966; Kriebel, 1975). Grossman *et al* (1973) used Golgi impregnation techniques to show that class A cells (presumed relay cells) are 15-20μm in diameter with myelinated axons while class B cells (presumed interneurones) are much smaller (about 10μm in diameter); Webster and Rowe (1984) showed using Golgi and HRP techniques that some cells in the rat dLGN do not have axons which project to the visual cortex. The authors concluded that these locally terminating cells are intrinsic inhibitory interneurones. The two classes of cells can also be distinguished physiologically according to the response to optic tract stimulation (Sumitomo and Iwama, 1977); this study showed that 94% of a sample were relay cells and 6%

interneurones. This ratio was constant throughout all regions of dLGN. More recently, Gabbott *et al* (1986) have shown using immunocytochemistry that 21% of neurones in the rat dLGN stain positively for GABA, suggesting the proportion of interneurones to be somewhat higher and not uniformly distributed.

I.1.(ii).b.2. Projections of the rat dLGN

The dLGN projects almost exclusively via the optic radiation to lamina IV and the lower part of lamina III of the primary visual cortex, with some less dense innervation of laminae I and VI and sparse innervation of the secondary visual cortical area (Ribak and Peters, 1975; Hughes, 1977). This is in contrast to the cat, whose dLGN also has an important projection to the secondary visual cortex (area 18), but similar to other animals, including the primate.

FIGURE 2: *The neuronal connections of the rat dorsal lateral geniculate nucleus*

(overleaf)

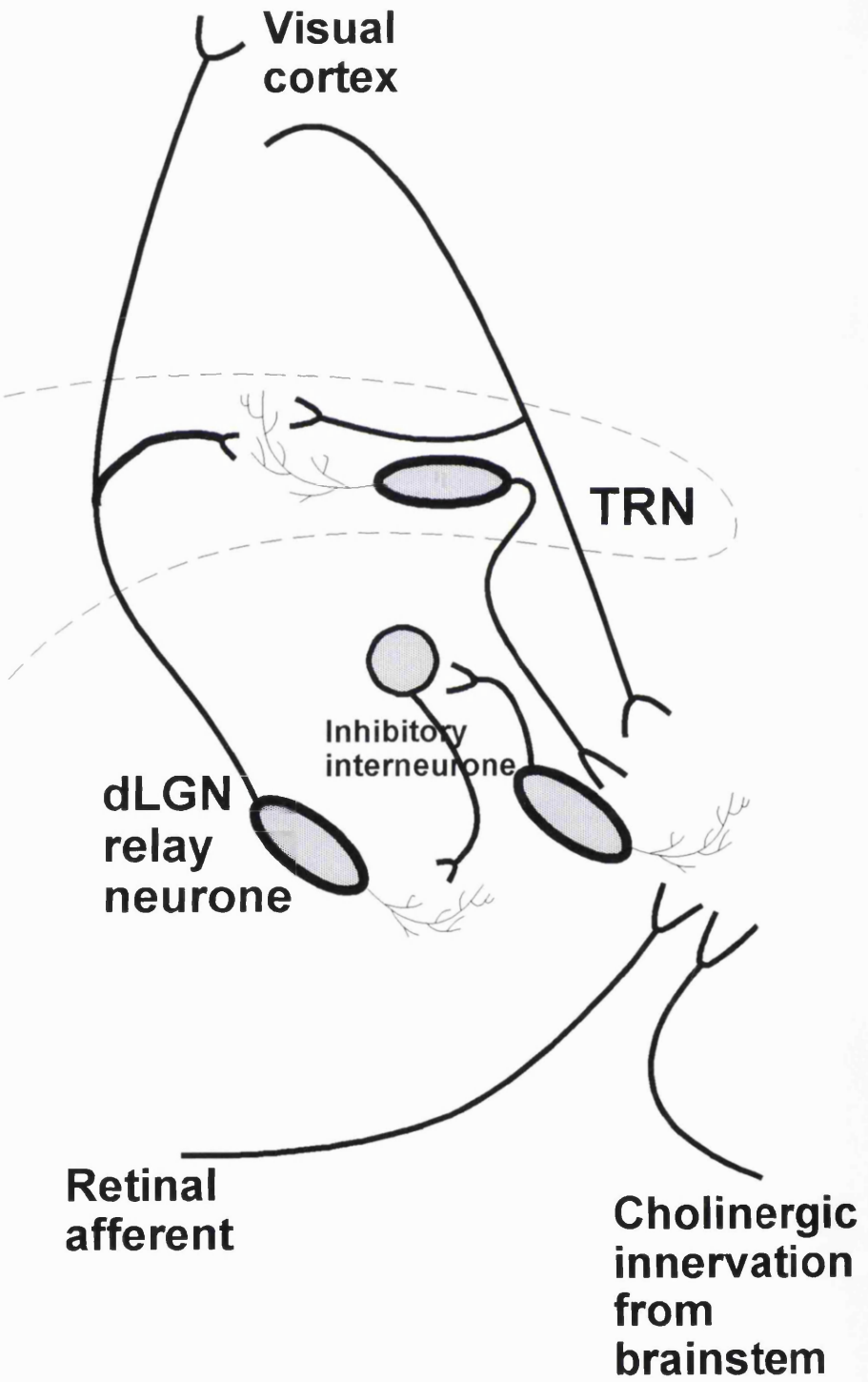

Visual cortex
TRN
Inhibitory interneurone
dLGN relay neurone
Retinal afferent
Cholinergic innervation from brainstem

FIGURE 2

The neuronal connections of the rat dorsal lateral geniculate nucleus

This figure schematically represents the circiutry and connections of the rat's dorsal lateral geniculate nucleus; for further details, see section I.1.(ii).b.

I.1.(iii). THE BIOPHYSICAL PROPERTIES OF THALAMIC CELL MEMBRANES

Many of the properties of thalamic cells can be explained in terms of the intrinsic properties of their cell membranes, which appear to be ubiquitous within the dorsal thalamus (i.e. membrane biophysical properties function similarly in all dorsal thalamic nuclei) and which in some cases override the effects of ligand-gated ion channels (Jahnsen and Llinás, 1984a&b).

Thalamocortical relay cells possess two distinct modes of firing: oscillatory (or burst firing) mode and tonically activated (or single spike) mode. The dynamics of the changeover from one mode to the other are dependent upon the interplay between conductances of the cell membrane, which in turn are influenced by brainstem innervation and the TRN [Steriade *et al*, 1985; see also section 1.(iv)].

Tonically activated mode occurs at membrane potentials positive to -55mV (in *in vitro* cells whose resting potential was -64 $\pm$ 5mV) (Jahnsen and Llinás, 1984a&b) and consists of a faithful thalamocortical action potential representation of the stimulation pattern. At these potentials, in addition to the conventional Na^+ and K^+ conductances, the thalamocortical response is mediated by a persistent Na^+ current ($I_{Na.p}$), activated following a depolarising current pulse which causes a `plateau' depolarisation which is capable bringing the membrane into the range of potentials which supports the generation of Na^+-mediated action potentials in the conventional, rapidly inactivating manner (Jahnsen and Llinás, 1984a). $I_{Na.p}$ has a lower activation threshold than the conventional Na^+ action potential. During such periods, each action potential is followed by an after-hyperpolarisation (AHP); Jahnsen and Llinás (1984b) described an AHP which hyperpolarised the membrane by around 15mV below the firing potential and which lasted for 100ms. The AHP is mediated in the main by a K^+ conductance which is

dependent on the presence of Ca^{2+} (demonstrated by replacement of Ca^{2+} in the bathing medium with alternative divalent cations), while an additional non-Ca^{2+}-dependent fast transient K^+ conductance (I_A) is responsible for the return of the membrane potential to baseline (Jahnsen and Llinás, 1984b). It has been suggested with some justification that $I_{Na.p}$ is responsible for opposing the effects of the AHP and as such to enable thalamocortical neurones to function at a higher frequency of firing (Steriade and Deschênes, 1984).

The contrasting situation is the oscillatory mode, which can be explained in terms of the low threshold spike (LTS or I_t) which is Ca^{2+} mediated. The LTS is inactivated at membrane potentials positive to -55mV, but becomes deinactivated if the membrane potential is held negative to -65mV for a short time (around 200ms). Following deinactivation, a small depolarisation which follows the offset of a hyperpolarising current pulse (or an IPSP) activates the LTS, resulting in a prolonged Ca^{2+} spike, lasting 20-30ms. Superimposed upon the LTS are 2-8 high frequency (200-400Hz) fast Na^+ action potentials which are triggered by the Ca^{2+} induced depolarisation (Jahnsen and Llinás, 1984a; McCormick and Feeser, 1990; von Krosigk *et al*, 1993). This mechanism is also responsible for the generation of rebound spikes, a phenomenon which can be observed following a short stimulus from which a few action potential spikes are evoked: IPSPs evoked from the TRN hyperpolarise the relay cell membrane into the range of potentials in which I_t is de-inactivated, resulting in an LTS and a burst of action potentials. This occurs both in VB (Salt and Eaton, 1991b) and in LGN (at least in Y cells) (Lo *et al*, 1991).

McCormick and Pape (1990a) have reported the influence of a hyperpolarisation-activated mixed monovalent cation inward current (I_h) on the mode of firing of

thalamocortical neurones. I_h is activated at potentials negative to -60mV, and causes a slight depolarisation of the membrane. Thus, I_h contributes to maintaining the membrane potentials which favour tonic firing rather than burst discharges. Soltesz *et al* (1991) have further emphasised the role of this current in the control of thalamocortical oscillatory activity.

The two modes of firing of thalamocortical neurones are clearly linked with the state of arousal and are evident during electroencephalographic (EEG) recording. During periods of attentiveness or rapid eye movement sleep (EEG-desynchronised periods), thalamocortical neurones function in the tonically activated mode. In periods of sleep or anaesthesia, however, the EEG switches to a synchronised pattern and likewise thalamocortical cells enter oscillatory mode. (See Steriade, *et al* (1993) for review).

Explanation, therefore, can be found for the action of the brainstem cholinergic and noradrenergic input to the thalamus. McCormick (1992) has demonstrated that application of acetylcholine or noradrenaline to thalamocortical relay neurones results in the reduction of an outward K^+ conductance, causing a depolarisation of the membrane and hence favouring the tonically activated mode of firing. In this way the brainstem may be responsible for maintaining the level of arousal of higher CNS structures by ensuring that thalamocortical information transfer proceeds with high fidelity. Further support for this suggestion can be found in the work of Steriade *et al* (1990) who showed a link between activity in the cholinergic neurones of the brainstem, the EEG phase and the mode of thalamocortical firing. Brainstem influences on thalamic neurotransmission are discussed below [section I.1.(iv)].

I.1.(iv). THE INFLUENCE OF THE BRAINSTEM ON THALAMIC SENSORY NEUROTRANSMISSION

I.1.(iv).a. *Cholinergic input*

The thalamus is extensively innervated by cholinergic neurones from the pedunculopontine and lateral dorsal tegmental nuclei of the brainstem; as well as staining for choline acetyltransferase (a marker of the synthesis of acetylcholine) these neurones are also positive for NADPH-diaphorase (Mackay-Sim *et al*, 1983; Levey *et al*, 1987; Fitzpatrick *et al*; 1989; Bickford *et al*, 1993). Several workers have attempted to mimic the action of these neurones by applying acetylcholine to thalamic cells *in vivo*; the result in sensory nuclei is an excitation (McCance *et al*, 1968 a&b; Sillito *et al*, 1983; Eysel *et al*, 1986), while in the TRN (or PGN) the result is an inhibition of firing (Sillito *et al*, 1983). Similarly, *in vitro*, McCormick and Prince (1987) found that application of acetylcholine to feline thalamic relay cells caused a depolarisation and a switch to single spike activity and away from rhythmic burst firing (McCormick, 1992). The action of acetylcholine on thalamic neurones represents a combination of two effects, one mediated by nicotinic receptors and one mediated by muscarinic receptors. The nicotinic effect is a rapid depolarisation caused by an increase in inward cation current, while the muscarinic effect is a slower, longer lasting depolarisation caused by decrease in an outward K^+ conductance. In some thalamic nuclei of some species (not in the dLGN of the rat) there is also a muscarinic receptor-mediated hyperpolarising current carried by K^+ (McCormick and Prince, 1987; McCormick, 1992). McCormick and Pape (1988) demonstrated that in the dLGN of the cat, acetylcholine has a further action, that being an inhibition of GABAergic inhibitory interneurones to cause a net excitation of relay cells. This is achieved by increasing a membrane K^+ conductance and is mediated via

muscarinic receptors. The effect of acetylcholine on GABAergic interneurones is analogous to that on neurones of the TRN (or PGN); these cells constitute an inhibitory feedback loop with thalamocortical relay cells, and are inhibited by the application of acetylcholine, thus facilitating thalamocortical transmission (Sillito *et al*, 1983). Steriade *et al* (1990) tested the hypothesis that the brainstem cholinergic input to the thalamus is involved in the control of the processes which govern attentiveness and the integrity of thalamocortical neurotransmission; this study concluded that by means of a direct cholinergic depolarisation, the brainstem cholinergic input to the thalamus was responsible for the maintenance of thalamocortical neurones in the tonically activated mode associated with EEG desynchronisation and for decreasing the probability of burst firing and spindle oscillations. Uhlrich *et al* (1995) studied the effects of stimulation of the brainstem parabrachial region on the dLGN of the cat in order to observe any changes in receptive field properties; in addition to a generalised enhancement of the dLGN cells' responsiveness, the results showed an enhancement of the centre/surround inhibition properties of dLGN cells such that a mechanism for the adjustment of contrast sensitivity may be under cholinergic influence.

The cholinergic input to the thalamus has recently been shown to be of even greater importance than was previously thought, given that the enzyme for the synthesis of nitric oxide is colocated in these neurones (Bickford *et al*, 1993). The implications of this are discussed in section 4.(vii).

I.1.(iv).b. *Noradrenergic input*

The locus coeruleus is the origin of the noradrenergic innervation of the thalamus (Ahlsen and Lo, 1982); stimulation of the locus coeruleus results in an enhancement of

responses in dLGN (Rogawski and Aghajanian, 1980a, b& c; Kayama 1985) caused by a slow depolarisation mediated by α_1-adrenoceptors and a decreased K^+ conductance (McCormick and Prince, 1988). In addition, ß-adrenoceptors mediate an effect on the hyperpolarisation-activated cation current I_h, such that its activation occurs at more positive membrane potentials (McCormick and Pape, 1990a&b; McCormick, 1992). McCormick and Pape (1990b) suggest that this effect may be exerted through an adenylate cyclase/cyclic AMP/cyclic AMP-dependent protein kinase system; they point out that the functional consequence of this effect on I_h would be an increased current amplitude at rest and as such a slight depolarisation of the membrane, resulting in a shift of the membrane potential away from the range in which I_t could be activated and so to decrease the probability of burst discharges.

I.1.(iv).c. *Serotonergic input*

In addition to cholinergic and noradrenergic inputs, the thalamus also receives input from the dorsal raphe nuclei using 5-hydroxytryptamine (5-HT) as a neurotransmitter (Ahlsen and Lo, 1982). *In vivo* stimulation of the dorsal raphe causes an inhibition of responses in dLGN (Kayama *et al*, 1989), although it has been hypothesised that this action is an indirect effect, resulting from stimulation of GABAergic inhibitory interneurones (McCormick and Pape, 1990b), as *in vitro* application of 5-HT to relay neurones in dLGN causes a small depolarisation, rather than an inhibitory effect.

SECTION I.2

THE PHARMACOLOGY OF EXCITATORY AMINO ACID RECEPTORS AND THEIR RELATION TO THALAMIC NEUROTRANSMISSION

I.2.(i). RECEPTORS

There are three broad divisions of excitatory amino acid (EAA) receptors, all of which are involved in thalamic neurotransmission. Two of these divisions (NMDA [N-methyl-D-aspartate] receptors and non-NMDA receptors) are ionotropic receptors, that is they allow the opening of an integral ionophore and the passage of ionic current. The third broad category, the metabotropic glutamate receptors (mGluRs) utilise an alternative second messenger system via linkage to a G-protein, i.e. a linkage to adenylate cyclase or to the metabolism of inositol phosphates.

I.2.(i).a. *NMDA receptors: pharmacology*

Of the three categories of EAA receptor, the most extensively researched is the NMDA receptor, that is, receptors which are selectively activated by the aspartate analogue NMDA and competitively antagonised by D-2-aminophosphonopentanoic acid (AP5) and 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP). These receptors possess multiple sites for pharmacological interaction, which can be summarised as follows:

I.2.(i).a.1. *The strychnine-insensitive glycine site* must be occupied by glycine to facilitate opening of the ion channel in response to binding of NMDA or aspartate or glutamate (Thomson, 1990); antagonists at this site (such as 7-chlorokynurenate) prevent

normal function of the receptor (Kemp *et al*, 1988). Measured concentrations of glycine in the CNS suggest that the site would be permanently occupied (Kemp and Leeson, 1993), but this may have led to the function of the glycine site being underestimated. Recent authors (Schell *et al*, 1995) have suggested a role for glial cells as sites for glycine (or D-serine) release following activation by NMDA receptors which allied with a mechanism for maintaining the synaptic glycine concentration at a low level may represent a means of modulating NMDA receptor function; glycine transporters are extensively present in the CNS and appear to be colocalised in areas where NMDA receptors are expressed. Salt (1989) demonstrated a potentiating effect of D-serine on NMDA receptor-mediated thalamic sensory responses *in vivo*, strongly indicating that physiologically the glycine site of the NMDA receptor is unsaturated. Similarly, antagonists at this site have inhibitory actions on NMDA receptor-mediated responses in the thalamus (Salt, 1989). D-Serine has been identified as being present in the central nervous system, and given that it appears to be localised to glial cells, its release may be controlled in such a way as to modulate NMDA receptor function (Schell *et al*, 1995).

Binding of glutamate to its site on the receptor appears to influence the binding of glycine by an allosteric mechanism (Benveniste *et al*, 1990) with the result that the affinity of the glycine site for its ligand is reduced. This could conceivably represent a mechanism by which the opening time of the ionophore is minimised.

I.2.(i).a.2. *The phencyclidine binding site* is the locus of action of several non-competitive, use-dependent antagonists, including phencyclidine itself, MK-801 and the general anaesthetic ketamine (Wong *et al*, 1986). This site is located within the ion channel (hence its use-dependency), but is distinct from the Mg^{2+} site (Harrison and Simmonds, 1985, and see below).

I.2.(i).a.3. *The magnesium binding site* is located within the ionophore itself, and is thought to be the explanation for the unusual voltage-dependent properties of the NMDA receptor (Mayer *et al*, 1984). It is hypothesised that membrane depolarisation (perhaps due to activation of another type of receptor) enables Mg^{2+} to leave the channel and allow current to pass (Ascher and Nowak, 1988). This voltage dependency combined with ligand gating may be an important mechanism for the integration of information within the nervous system, such that the NMDA receptor is capable of combining two inputs.

Some recent evidence has thrown doubt upon the suggestion that Mg^{2+} is always present in the NMDA receptor's ion channel. It now seems more likely that the magnesium blockade only occurs in certain subunit conformations and certain brain regions (Monyer *et al*, 1994).

I.2.(i).a.4. *The polyamime site* is another modulatory site, which when occupied by its endogenous agonist spermine enhances responses to NMDA (Radpour and Thomson, 1990). Spermine and spermidine have a complex action consisting of both inhibitory and excitatory actions. The inhibitory action is mediated at least in part by allosteric modification of an area within the ionophore, such as the Mg^{2+} site; the excitatory action results from an increase in the affinity of the glycine site for its ligand (Benveniste and Mayer, 1993).

I.2.(i).a.5. *A Zn^{2+} site*, distinct from the Mg^{2+} site, has been proposed (Mayer *et al*, 1989); at this site, Zn^{2+} ions appear to antagonise responses to NMDA non-competitively, but this action is poorly understood.

I.2.(i).a.6. *A redox modulatory site*, which is thought to be involved in the potentiation of the responses to NMDA observed with sulphydryl reducing agents such as

dithiothreitol (DTT) and conversely the inhibition produced by oxidising agents such as 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The mechanism of redox alterations in NMDA receptor function appears to require formation or breaking of disulphide bonds between adjacent thiol groups on the extracellular side of the receptor, causing a long-lasting modification (Aizenman *et al*, 1989, Tang and Aizenman, 1993, and see below). The redox modulatory site appears to be subunit specific; Köhr *et al* (1994) found that receptors expressing certain subunits were most susceptible to potentiation by DTT.

I.2.(i).b. *Molecular biology of NMDA receptors*

NMDA receptors are constructed from combinations of two subunits, NR1 and NR2 (also referred to as NMDAR1 and NMDAR2), each of which exist in multiple forms (there are 4 subtypes of NR2, each of which may have splice variants, and 8 splice variants of NR1). Native NMDA receptors are constructed from one of the NR1 splice variants plus one of the NR2 subunits, although it is possible to artificially assemble homomeric receptors from NR1 subunits alone and express them in *Xenopus* oocytes (McBain and Mayer, 1994).

The type of NR2 subunit (i.e. whether NR2A, NR2B, NR2C or NR2D) appears to be crucial in the determination of the receptor's pharmacological properties, for example, combinations of NR1/NR2C have a lower conductance than NR1/NR2A or NR1/NR2B (Stern *et al*, 1992). Receptors expressed to contain the NR2C subunit also have a lower affinity for Mg^{2+} (Monyer *et al*, 1992). Homomeric NR1 receptors appear not to be susceptible to redox modulation by DTT or DTNB; it has been reported that the presence of the NR2A subunit confers the receptor with the capability to be modulated to a greater extent by reducing agents, while the same study also suggests that

this subunit allows a greater degree of spontaneous re-oxidation following the potentiating effects of DTT. As a result it has be suggested that the NR2A subunit has two discrete redox modulatory sites, while NR2B and NR2C have only one (Köhr *et al*, 1994).

The various configurations of NMDA receptors are differentially distributed through the CNS; NR2D is mostly expressed during development (Watanabe *et al*, 1992). In the thalamus, NR2A and NR2B are both present (Buller *et al*, 1994; Monyer *et al*, 1994).

I.2.(ii).a. *Non-NMDA (AMPA/kainate) ionotropic receptors*

This group of ionotropic excitatory amino acid receptors was classified as distinct from NMDA receptors by virtue of their sensitivity to the agonists quisqualate or α-amino-4-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate, and not to NMDA. They are antagonised by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX) (Watkins *et al*, 1990). Like NMDA receptors, non-NMDA receptors are built from subunits: these are termed GluR1, GluR2, GluR3, and GluR4 (the AMPA receptor subunits, also called GluR A-D) plus the kainate subunits GluR5, GluR6, GluR7, KA1 and KA2. Each of the AMPA receptor subunits can exit in two alternative splice forms (the so-called `flip' and `flop' forms); the flip form appears to produce a greater current amplitude in response to glutamate than the flop variant (Sommer *et al*, 1990).

Any of these subunits can form homomeric complexes or heteromeric complexes with one other member of its group (Seeburg, 1993; Hollmann and Heinemann, 1994).

Of the AMPA subunits, GluR2 is exceptional in that it is relatively less permeable to Ca^{2+} than the other subunits, and has a linear current-voltage relationship plot (the other subunits are inward-rectifiers) (Verdoorn *et al*, 1991). The low Ca^{2+} permeability and relative non-rectification of GluR2 is attributable to a site in its second transmembrane domain which has been termed the Q/R site, representing a single amino acid. In GluR2 subunits, the Q/R site residue is arginine (R), carrying a positive charge, while in other subunits it is glutamine (Q). However, if this residue is replaced with asparagine the result is a linear current-voltage relationship while retaining a high Ca^{2+} permeability, suggesting that the two phenomena are controlled by different factors. The NMDA receptor has an asparagine residue in its second transmembrane domain, and has the properties of the AMPA receptor which has been mutated at this site, i.e. relatively high Ca^{2+} permeability and a linear current-voltage relationship.

In addition, the GluR2 RNA undergoes a process of editing. The gene for GluR2 appears to code for a glutamine residue at the Q/R site, i.e. to code for high Ca^{2+} permeability. A highly specific RNA editing mechanism adjusts the coding sequence such that glutamine is replaced with arginine. The possibility arises, therefore, that neurones are able to express GluR2 in two alternative forms, one of high Ca^{2+} permeability and one of lower Ca^{2+} permeability (Sommer *et al*, 1991).

In situ hybridisation studies reveal differential distribution of the subunit types: GluR2 is found throughout the CNS, but, for example, GluR2 and GluR4 are found in cerebellar granule cells, but GluR1 and GluR3 are not. Of this group, GluR4 is especially abundant in the thalamus, but all are expressed in the thalamic reticular nucleus (Keinänen *et al*, 1990).

I.2.(ii).b. *GluR5, GluR6 and GluR7: Low affinity kainate receptor subunits*

When expressed in functional receptors, GluR5-7 are selective for kainate over AMPA and have a lower amino acid homology with GluR1-4 than with each other. The same Q/R site RNA editing mechanism as in GluR2 is present when GluR5 or GluR6 are expressed, but not GluR7. The result of the amino acid substitution is the same as is the case for GluR2, i.e. a change in the amplitude of response and of the rectification properties of the channel. However, unlike GluR2, the editing remains incomplete such that cells are able to express both edited and unedited, low Ca^{2+} conductance and high Ca^{2+} conductance forms of the receptor (Sommer *et al*, 1991). GluR6 has also been proved to possess additional RNA editing sites (Köhler *et al*, 1993), while GluR5 has several different slice variants (Sommer *et al*, 1992).

I.2.(ii).c. *KA1 and KA2: High affinity kainate receptor subunits*

Studies of amino acid sequence have revealed a subfamily of kainate-sensitive receptors distinct from GluR5-7 with a high affinity for kainate (Lomeli *et al*, 1992). These subunits show no splice variation and possess no means of Q/R site RNA editing. They do not function when expressed as homomeric receptors but KA2 subunits do form functional channels when coexpressed with GluR5 or GluR6 (Herb *et al*, 1992). The localisation of these receptor subunits in the thalamus is discussed below (section I.2.(iv).).

I.2.(iii). METABOTROPIC GLUTAMATE RECEPTORS (mGluRs)

The eight G-protein linked metabotropic glutamate receptors can be subgrouped as follows according to their intracellular transduction mechanisms and

agonist/antagonist selectivity (Nakanishi, 1992; Watkins and Collingridge, 1994; Pin and Duvoisin, 1995).

Group I: mGluR1, mGluR5
Coupled to inositol phosphate metabolism

Group II: mGluR2, mGluR3
Negatively coupled to adenylate cyclase/cAMP

Group III: mGluR4, mGluR6, mGluR7, mGluR8
Negatively coupled to adenylate cyclase/cAMP,
but with anagonist selectivity profile different
to Group II receptors.

The mGluRs represent an expanding field of research and as such the above list fails to explain all the reported properties of these receptors and is almost certainly incomplete. All groups are found in both pre- and postsynaptic locations, although Group I receptors are predominantly postsynaptic and Groups II and III are predominantly presynaptic.

The glutamate analogue (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD] is an agonist at mGluR1, mGluR2, mGluR3 and mGluR5. L-2-amino-4-phosphonobutanoate (L-AP4) is an agonist at mGluR4, mGluR6, mGluR7 and mGluR8; 2S,1'S,2'S-2-(2'-carboxycyclopropyl)glycine (L-CCG-I) is relatively selective for mGluR2 and mGluR3. The lack of selectivity of any of these agonists for any

particular receptor subtype has hindered the progress of research into the complex roles of mGluRs in which more than one subtype may be involved. However, several recently developed antagonists have proved successful in the elucidation of metabotropic receptor function and should continue to clarify the situation (Watkins and Collingridge, 1994, Salt and Eaton, 1994, Roberts, 1995).

I.2.(iv). THE LOCATION AND FUNCTION OF GLUTAMATE RECEPTORS IN THE THALAMUS

I.2.(iv).a. *Ionotropic receptors*

The relay nuclei of the thalamus appear to be positive for the NMDA receptor subunits NR1, NR2A and NR2B. NR1 subunits have been pinpointed to postsynaptic dendrites of relay neurones, to thalamic reticular nucleus neurones and also to presynaptic sensory afferent neurones. Thalamic glial cells do not stain for NMDA receptor subunits (Petralia *et al*, 1994a, Petralia *et al*, 1994c).

The AMPA receptor subunits GluR2 and GluR3 are present postsynaptically in thalamic relay neurones, but GluR1 appears not to be expressed and GluR4 is only found in the TRN (Spreafico *et al*, 1994). The distribution of kainate-sensitive receptors is less clear, but Petralia *et al* (1994b) have reported the immunostaining of some parts of the thalamus for GluR6, GluR7 and KA2. Thalamic astrocytes stain positively for GluR1 and GluR4, in contrast to relay cells.

I.2.(iv).b. *Metabotropic receptors*

mGluRs are expressed in great numbers in the thalamus, with mGluR1 and mGluR5 especially prevalent (Shigemoto *et al*, 1992; Martin *et al*, 1992; Romano *et al*,

1995). mGluR4 is also present (Tanabe *et al*, 1993). Ohishi *et al* (1993) and Tanabe *et al* (1993) have reported expression of mGluR3 in the thalamic reticular nucleus.

I.2.(v). NEUROTRANSMISSION IN THE THALAMUS

I.2.(v).a. *Retino/lemnisco/trigemino-thalamic transmission*

Ascending sensory neurotransmission through the ventrobasal complex and the dorsal lateral geniculate nucleus is mediated by a transmitter which utilises excitatory amino acid receptors of both the NMDA and non-NMDA subcategories (Salt, 1987; Sillito *et al*, 1990a&b; Salt and Eaton, 1991b). It seems likely that the excitatory neurotransmitter is glutamate, which has been identified in lemniscal afferents to VB (De Biasi and Rustioni, 1990; Hamori *et al*, 1990; De Biasi *et al*, 1994) and retinal afferents to dLGN (Montero and Wenthold, 1989) although other putative transmitters have been implicated, particularly in retino-geniculate transmission, including L-homocysteate (HCA) and N-acetylaspartylglutamate (NAAG); NAAG is known to be present in retinal ganglion cells (Moffet *et al*, 1991) although the hypothesis of an action of this dipeptide via EAA receptors is thought to be unlikely (Henderson and Salt, 1988; Jones and Sillito, 1992). HCA has also been suggested to be a candidate in this role (Jones and Sillito, 1992), but is known to be localised in glial rather than neuronal elements (Grandes *et al*, 1991).

Studies employing selective antagonists have revealed greater detail regarding the physiology of thalamic neurotransmission and have enabled the roles of NMDA and non-NMDA ionotropic receptors to be distinguished. Salt (1986; Salt, 1987) reported that in the ventrobasal complex of the rat sensory responses of relay neurones are initially mediated by receptors which are sensitive to non-NMDA ionotropic receptor

antagonists, followed in cases of sustained stimulation by a greater involvement of receptors susceptible to blockade by NMDA receptor antagonists. Similarly, Salt and Eaton (1991b) used an intracellular recording preparation to demonstrate relay cell EPSPs in which an early phase was sensitive to the non-NMDA antagonist CNQX and a later phase was sensitive to the NMDA receptor antagonist CPP. In the dLGN of the cat, both transient and sustained visual responses are able to be antagonised by both CPP and CNQX, suggesting that both NMDA and non-NMDA receptors are involved at all stages of the response in all classes of dLGN relay cell (Sillito *et al*, 1990a&b). However, Turner *et al* (1994), using intracellular recording techniques, revealed a frequency dependent role of NMDA and non-NMDA receptors in dLGN such that a fast EPSP is mediated by solely non-NMDA receptors and a smaller and slower potential, resistant to CNQX antagonism, is mediated by NMDA receptors.

In addition to the ionotropic EAA receptors, recent work has uncovered an involvement for metabotropic glutamate receptors in transmission in the ventrobasal complex; Salt and Eaton (1991a) showed a direct excitatory action of the non-selective mGluR agonist 1S,3R-ACPD and later used phenylglycine-derived mGluR antagonists to show Group I mGluRs (probably mGluR1) are involved in nociceptive VB transmission (Eaton *et al*, 1993; Salt and Eaton, 1994); mGluR1 receptors are known to be abundant on postsynaptic dendrites in VB (Martin *et al*, 1992 and see above).

I.2.(v).b. *Corticothalamic neurotransmission*

Studies of corticothalamic neurotransmission appear to implicate the NMDA subclass of receptors in this role; Deschênes and Hu (1990) demonstrated that corticothalamic excitation could be attenuated by NMDA receptor antagonists following

ablation of the thalamic reticular nucleus (whose input would have obscured the results). These results were confirmed by Eaton and Salt (1996) in the rat ventrobasal complex using iontophoretic application of CPP; application of (S)-4-carboxyphenylglycine (S4CPG, a Group I metabotropic receptor antagonist) was also shown in the same study to reduce the corticothalamic EPSP amplitude, implying a role for Group I metabotropic glutamate receptors in corticothalamic excitation. Moreover, McCormick and von Krosigk (1992) observed a depolarisation of thalamic neurones following application of *trans*-ACPD similar to that which is seen during corticothalamic EPSPs, suggesting that cortical input to the thalamus may be mediated at least in part by metabotropic glutamate receptors, which have been identified postsynaptically in the thalamus (Martin *et al*, 1992; Godwin *et al*, 1995). Metabotropic receptors of the mGluR1 type have been implicated the most directly in this role (Godwin *et al*, 1996; Vidnyanszky *et al*, 1996). Corticothalamic input appears to be crucial to thalamic function (Sillito *et al*, 1993; Sillito *et al*, 1994) but remains relatively under-researched due to the technical complications of collateral stimulation of the TRN.

I.2.(v).c. *Presynaptic factors*

Recent work by Salt and Eaton (1995) has identified a role for mGluRs in presynaptic modulation of thalamic sensory neurotransmission. This study identified a reduction in the amplitude of IPSPs and a reduction of the degree of inhibition seen in a condition-test paradigm following iontophoretic application of the mGluR agonists L-AP4 and CCG-I. Given the absence of intrinsic GABAergic inhibitory influence in the ventrobasal thalamus, the authors concluded that the metabotropic agonists must be acting on terminals of neurones whose origin is in the thalamic reticular nucleus.

Moreover, the same study applied antagonists to block the disinhibitory action of the agonists and drew some conclusions regarding the identity of the receptor subtypes involved: these appear to be mGluRs of Group II or Group III. This research is at an early stage and the physiological significance of the presynaptic modulation of TRN influence on relay nuclei remains to be established.

I.2.(v).d. *GABAergic inhibitory connections*

The ventrobasal complex of the rat is exceptional in that it has no identified Golgi Type II inhibitory interneurones [see section 1.(ii).a]. The dorsal lateral geniculate nucleus of the rat and the VB complex and dLGN of higher species all possess intrinsic inhibitory interneurones which are GABAergic (Ohara *et al*, 1983; Madarasz *et al*, 1985; Bentivoglio *et al*, 1986; Ohara and Lieberman, 1993); in all cases the sensory relay nuclei receive GABAergic inhibitory influence from the thalamic reticular nucleus (De Biasi *et al*, 1988). The pharmacology of the input to intrinsic inhibitory neurones is not well established, but the TRN has been more extensively studied: De Curtis *et al* (1989) demonstrated the involvement of both NMDA and non-NMDA ionotropic EAA receptors in the response of TRN cells to stimulation of cortical afferents. Some subtypes of mGluRs have also been identified in TRN, most notably mGluR3 (Tanabe *et al*, 1993, and see above).

SECTION I.3

THE ROLE OF GLUTATHIONE IN THE CENTRAL NERVOUS SYSTEM

Glutathione is a tripeptide, consisting of residues of glutamate, cysteine and glycine (it could also be described as L-gamma-glutamyl-L-cysteinylglycine), all of which are neuroactive in their own right. It is well established that glutathione is present in relatively high concentrations (in the millimolar range) in the nervous system of the rat (Reichelt and Fonnum, 1969; Kirstein *et al*, 1991) and that it is present both in neurones and glial cells (Slivka *et al*, 1987a) although there is some dispute as to the relative distribution between neuronal elements and non-neuronal cells. It is a molecule with a multitude of functions, too diverse for discussion in this context (for example as an antioxidant, as a coenzyme, as a conjugate for toxins), which are reviewed by Meister and Anderson (1983).

Glutathione is synthesised in a two stage process by two enzymes: gamma-glutamylcysteine synthetase [which is rate limiting and which can be irreversibly inhibited by buthionine sulphoximine and related compounds (Griffith and Meister, 1979)] and GSH synthetase, and broken down by gamma-glutamyl transpeptidase. It is mostly present intracellularly, under normal circumstances predominantly (99%) in its reduced form (GSH) although the oxidised, dimeric form (GSSG or glutathione disulphide) is also present and increases in its relative proportion during periods of oxidative stress. GSH can be converted to GSSG by a number of different enzymes, but perhaps most crucially for its function in the CNS, GSH reacts with free radicals to form GSSG. The reverse reaction is catalysed by GSSG reductase (Meister and Anderson, 1983).

In the CNS, GSH has been suggested to be involved in synaptic transmission in

a number of roles (see for example Shaw *et al*, 1996), and its release into the extracellular environment has been detected (Zängerle *et al*, 1992). Selective binding sites for GSH have been described (Ogita and Yoneda, 1987; 1988; 1989) with a dissociation constant which could be relevant to the range of extracellular concentrations of GSH reported by Zängerle *et al* (1992). These bindings do not involve the formation of disulphide bonds between GSH molecules and membrane molecules, as they can be displaced or prevented by GSH analogues such as methyl-GSH; nor are they due to the release of glutamate from the GSH molecule which occurs during the incubation. These findings are suggestive of a role for GSH as a neurotransmitter or neuromodulator in its own right. Further evidence for this hypothesis comes from the finding that a large proportion (35%) of total brain glutathione is present in the synaptosomal fraction (Reichelt and Fonnum, 1969). Guo and Shaw (1992) and Guo *et al* (1992) have still further emphasised this putative neurotransmitter/neuromodulator role for glutathione with their identification of receptors for glutathione on astrocytes in culture, linked to phosphoinositide metabolism. Recent work (Pasqualotto *et al*, 1996; Shaw *et al*, 1996) has uncovered glutathione's ability to stimulate Na^+ currents into cortical neurones, and it has been further proposed that in this instance, glutathione is acting to stimulate its own receptor-coupled Na^+ ionophore.

Glutathione in either form is also capable of displacing radioactively labelled L-glutamate from its binding sites, at physiologically relevant concentrations (Ogita *et al*, 1986) with a slow dissociation; furthermore, other studies (Yoneda *et al*, 1990) have observed a displacement of labelled CPP from the NMDA receptor, also at low concentrations. Thus there is evidence for glutathione binding sites, for its depolarisation-induced release (Zängerle *et al*, 1992) and for its ability to induce currents

in cortical neurones (Shaw *et al*, 1996). Taken together, these observations may suggest a neurotransmitter role for glutathione. Furthermore, Shaw *et al* (1996) point out that their observation of glutathione-induced Na^+ currents was not blocked by AP5, and therefore did not involve NMDA receptors (at least not directly, see below); they suggest the participation of a new and distinct Na^+ channel.

A further possible means by which GSH could exert an effect on synaptic transmission in the CNS is via an indirect action on NMDA receptors. GSH is capable of the sequestration of Zn^{2+} (as is cysteine) (Li *et al*, 1954), while, as discussed elsewhere, Zn^{2+} ions have a modulatory effect at NMDA receptors (Mayer *et al*, 1989). This remains a purely hypothetical effect, in which the inhibitory influence of Zn^{2+} could be removed from the NMDA receptor by the presence of GSH. The NMDA receptor is also susceptible to positive modulation by reducing agents such as dithiothreitol and negative modulation by oxidising agents [see for example Aizenman *et al* (1989) and section 2.(i).a.6]. On the basis of such work, NMDA receptors have been postulated to possess a `redox modulatory site' which could in theory be influenced by the redox potential within the synapse, which could in turn be determined by the availability of thiol compounds such as GSH (Aizenman *et al*, 1989; Tang and Aizenman, 1993).

Glutathione has been implicated in several disease states, notably Parkinson's disease, in which it is found to be almost completely absent from the substantia nigra (Perry *et al*, 1982). Furthermore, glutathione depletion by buthionine sulphoximine exaggerates the effects of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a model of Parkinsonism (Wuellner *et al*, 1996). It may be the case, therefore, that glutathione exerts complex effects, being both a neurotransmitter or neuromodulator, and in addition being a neuroprotective factor.

SECTION I.4

NITRIC OXIDE AND ITS ROLE IN NEUROTRANSMISSION

I.4.(i). IDENTIFICATION OF NITRIC OXIDE AS A PHYSIOLOGICAL MEDIATOR

The importance of nitric oxide (NO) as a biological messenger molecule has become apparent as a result of extensive research spanning the last decade. NO was initially identified in the periphery as a mediator of vasodilation: the properties of the substance present in vascular endothelium known as endothelium derived relaxing factor (EDRF) could be attributed to NO, and although there remains some suggestion to the contrary, it is now accepted by most workers that EDRF and NO are at least functionally one and the same (Palmer *et al*, 1987).

EDRF was identified by Furchgott and Zawadski (1980), who discovered that acetylcholine's ability to cause vascular relaxation was crucially dependent on the presence of vascular endothelial tissue. A variety of substances, other than acetylcholine, were found to have their effects mediated in this way, that is by an intermediary molecule released by endothelial cells. Griffith *et al* (1984) went on to establish that EDRF was a molecule with a particularly short half-life (a matter of a few seconds, at least *in vitro*) which was tonically released from endothelial tissue, being released at a greater rate following stimulation with acetylcholine. EDRF could be prevented from exerting its effects by the presence of haemoglobin or methylene blue (Martin *et al*, 1985). Its mode of action appeared to involve the stimulation of soluble guanylate cyclase to elevate intracellular levels of cyclic GMP (Rapoport and Murad, 1983). EDRF was found to be susceptible to inhibition by the presence of oxygen radicals; addition of superoxide

dismutase to EDRF generating preparations prolonged its effects (Gryglewski *et al*, 1986).

Ignarro *et al* (1987) were among the first to make the suggestion that EDRF may in fact be NO or a similar species. The evidence for this came partly from chemiluminescent techniques for the measurement of NO, which were shown to be sensitive to the presence of EDRF. Comparison of the effects of NO with the effects of EDRF in various preparations corroborated this observation; both were found to be of similar half-life (variation between studies can be explained in terms of the differing contributions to NO's inactivation made by oxygen and superoxide, which vary according to individual laboratory conditions) and with effects on vasculature and on platelet aggregation which were indistinguishable (Palmer *et al*, 1987; Radomski *et al*, 1987; Moncada *et al*, 1988). Both NO and EDRF were susceptible to inhibition by the same factors, notably haemoglobin, which is known to bind NO (Martin *et al*, 1986). NO was also known to cause elevations in cyclic GMP levels in a similar way to EDRF (Kukovetz *et al*, 1979). Taken together, the various observations regarding the properties of NO and EDRF make a strong case for their being identical molecules.

Despite the evidence described above, some authors have suggested the possibility that EDRF more accurately reflects the properties of a nitrosothiol compound such as S-nitrosocysteine than the properties of NO *per se* (Myers *et al*, 1990). The evidence to support this claim is on the whole less substantial and convincing than the evidence for EDRF as NO and has been disputed (Feelisch *et al*, 1994), but S-nitrosothiols may be of importance under some circumstances. Myers *et al* (1990) point out that the S-nitrosocysteine molecule effectively incorporates NO and may be capable of releasing it; the implications of this are that the stability of the molecule is enhanced

and as such the molecule could exert a more prolonged physiological effect, while conversely the thiol could act as a buffer for excess NO. In addition, incorporation of NO into a nitrosothiol could enable an uptake mechanism to operate, thus limiting the molecule's distribution or the time span of its influence.

Chemically, NO would appear to be a strange molecule for use in a biological system, being a gaseous compound with a short half-life. The molecule possesses an unpaired electron, and is therefore defined as a `radical', but is considerably less reactive than this term suggests: nitric oxide reacts with oxygen and with certain other species, but is not as reactive a radical as, for instance, the hydroxyl radical. The reaction with oxygen to produce nitrogen dioxide is rapid in air, but under physiological conditions (i.e. in solution) this reaction is severely slowed and cannot by itself account for the molecule's short physiological half-life; the fate of nitric oxide is somewhat more complicated, largely involving conversion to nitrite before oxidation by oxyhaemoglobin to form nitrate (Yoshida *et al*, 1980; Spagnuolo *et al*, 1987). NO also reacts with superoxide (O_2^-) to generate peroxynitrite, in a reaction which also liberates the potentially toxic hydroxyl radical (OH^-).

A further complication for the biochemistry of NO arises from the possibility of multiple redox forms of the NO species. Lipton *et al* (1993) have attempted to explain the multiple actions of nitric oxide in terms of its possible existence in an oxidised form (NO^+, nitrosonium ions) or a reduced form (NO^-, nitroxide ions). [This work will be discussed with reference to the neurotoxic/neuroprotective effects of NO in a later section]. These possibilities are yet to be fully explored, but it may be the case that S-nitrosothiol compounds could effectively act as NO^+ species during the process of transnitrosation in which the NO^+ species is transferred to a suitable nucleophile, such

as another thiol (Stamler *et al*, 1992a). Furthermore it has been suggested that the redox state of NO may influence its target interactions, for instance transnitrosation reactions can influence the activity of some enzymes (Stamler *et al*, 1992a; Stamler *et al*, 1992b).

Since the initial discovery of nitric oxide as a physiological mediator molecule for acetylcholine's vasodilatory properties, an enormous variety of additional roles has been described, such that the molecule appears to be almost ubiquitous. The most extensively described roles are the regulation of vascular tone (Furchgott and Zawadski, 1980), the cytotoxic properties of macrophages (Nathan and Hibbs, 1991), and, as will be detailed in a later section, a role in neurotransmission in the central nervous system (Garthwaite, 1991).

I.4.(ii). THE BIOSYNTHESIS OF NITRIC OXIDE BY NITRIC OXIDE SYNTHASE

Nitric oxide is synthesised from the amino acid L-arginine by a family of enzymes known as nitric oxide synthase (NOS) in a reaction which also yields L-citrulline. The biochemistry of this process has been reviewed in detail by Marletta (1993), and will be outlined in the following paragraphs.

Palmer *et al* (1988a&b) established that L-arginine was the precursor of NO, using radiolabelled [^{15}N]-L-arginine to demonstrate synthesis of [^{15}N]-NO from the amino acid's terminal guanidino nitrogen atoms. D-Arginine is not a substrate for this reaction, and analogues of L-arginine have successfully been developed for use as inhibitors of NOS [see section 4.(v).a.].

Nitric oxide synthase exists in two broad categories: (i) constitutive NOS, found in the brain and vascular endothelium, which is regulated by Ca^{2+} and calmodulin

(Förstermann *et al*, 1991) and (ii) an inducible form, found in macrophages. The constitutive form was first isolated by Bredt and Snyder (1990) from rat cerebellum, the calmodulin dependence becoming apparent when the enzyme became non-functional upon purification. Subsequently, molecular biological cloning techniques have enabled the enzyme in both its forms to be studied in some detail. These techniques have also revealed regional heterogeneity in the precise amino acid sequence and hence have led to subclassification of NOS isoforms.

All isoforms of NOS appear to require a number of cofactors. The enzymes have recognition domains for reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Bredt *et al*, 1991b). The same work also pointed out homology between the cloned NOS and cytochrome P450 reductase, which also possesses recognition sites for NADPH, FAD and FMN. Cytochrome P450 reductase acts as an electron donor for the hepatic cytochrome P450 family of detoxifying enzymes; it is presumed that the portion of the NOS molecule which shares similarity in its amino acid sequence with cytochrome P450 reductase fulfils a similar electron donating function in the synthesis of NO. NOS also requires the presence of tetrahydrobiopterin (BH_4) as a cofactor (Mayer and Werner, 1995), which has been suggested to have a redox role (Mayer *et al*, 1991; Hevel and Marletta, 1992) or a role in the dimerisation and stability of NOS, at least in the inducible form (Giovanelli *et al*, 1991; Tzeng *et al*, 1995).

NOS contains a haem moiety (Klatt *et al*, 1992) and as such is able to bind carbon monoxide; carbon monoxide inhibits the function of the enzyme, suggesting that the haem group is essential for NO synthesis (White and Marletta, 1992). NO also binds to the haem moiety, and negatively modulates its function, thus acting as a negative

feedback loop to inhibit formation of more NO (Rengasamy and Johns, 1993).

The brain (or type I, nNOS) and endothelial (or type III, eNOS) isoforms of NOS are described as constitutive to refer to the fact that, unlike the macrophage form (type II, iNOS), they do not require protein synthesis *de novo,* at least under normal circumstances. Rather, the intracellular Ca^{2+} concentration is what critically determines the rate of the enzyme's function, for instance in the central nervous system, the elevation of intracellular Ca^{2+} which occurs following activation of some excitatory amino acid receptors is the trigger for NO synthesis (Bredt and Snyder, 1990). This enables the enzyme and its product to act over a short time scale, such as is required in neuronal transmission. The macrophage form of NOS (which is also found in CNS glial cells; Murphy *et al*, 1993) is not normally expressed, but protein synthesis begins within a period of a few hours following exposure to certain cytokines, such as gamma-interferon, whereupon NO is synthesised in quantities sufficient to be toxic to its target cells. Despite its non-requirement for Ca^{2+}, the inducible form of NOS binds calmodulin with high affinity (Cho *et al*, 1992).

Most forms of NOS possess sites for phosphorylation; Bredt *et al* (1992) described the range of possible phosphorylation modulatory sites present on neuronal NOS, which includes sites for phosphorylation by protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, cGMP-dependent protein kinase and cAMP-dependent protein kinase. All these phosphorylations are negatively modulatory, and as such it could be hypothesised that cyclic GMP may be able to modulate its own synthesis through a feedback mechanism involving cGMP-dependent protein kinase.

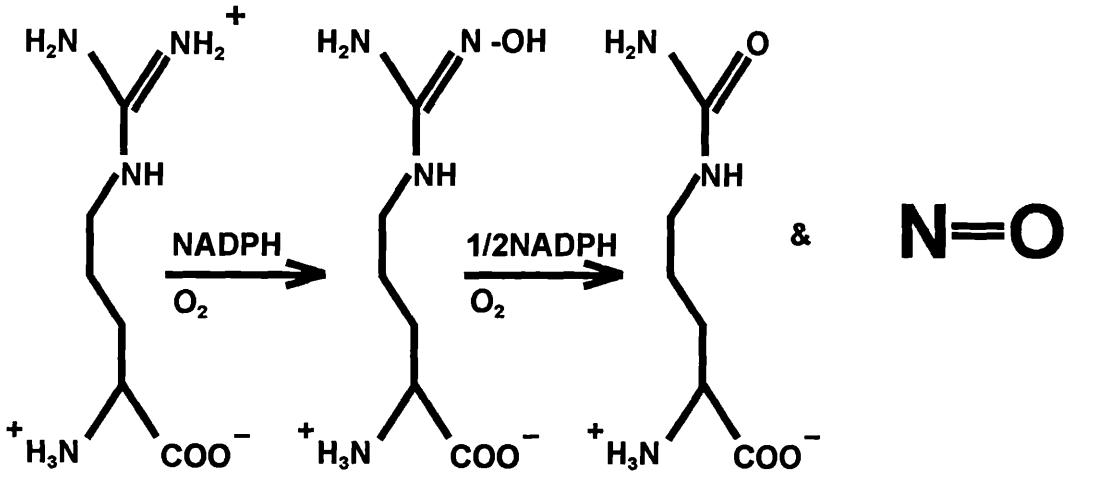
All isoforms of NOS exist as dimers; the neuronal form is of a higher molecular weight than the endothelial or macrophage forms, being 160kDa compared to 130kDa

(Bredt and Snyder, 1990; Schmidt *et al*, 1991). Unlike the particulate endothelial form, the neuronal form of NOS is present both as a soluble, cytosolic enzyme and in a particulate form (Hiki *et al*, 1993), suggesting a further subdivision of the NOS family.

The first step in the reaction catalysed by NOS is the N-hydroxylation of one of the guanidino nitrogen atoms of L-arginine, utilising NADPH and molecular oxygen, to form N^G-hydroxy-L-arginine (Stuehr *et al*, 1991). This step is followed by a number of other less well characterised steps, involving the haem moiety of NOS and additional NADPH (Korth *et al*, 1994). The end result is the generation of NO and L-citrulline. This reaction is summarised in the accompanying figure.

FIGURE 3: *Reaction scheme for the biosynthesis of nitric oxide from L-arginine*

(overleaf)



L-ARGININE

N-HYDROXY-L-ARGININE

L-CITRULLINE

NITRIC OXIDE

FIGURE 3

Reaction scheme for the biosynthesis of nitric oxide from L-arginine

This figure represents a summary of the steps in the conversion of L-arginine to L-citrulline and nitric oxide, i.e. the reaction catalysed by nitric oxide synthase. Cofactor requirements are indicated on the arrows.

I.4.(iii). NOS HISTOCHEMISTRY

The enzyme NADPH-diaphorase was first identified as being present in high concentrations in certain neurones by Thomas and Pearse (1964); a technique for histologically staining such neurones was based upon the reduction of tetrazolium salts to formazans which could be viewed microscopically. Hope *et al*, (1991) reappraised the importance of this work when their research made it apparent that neuronal NADPH-diaphorase is in fact a nitric oxide synthase. As a consequence, it has been proposed that neuronal NADPH-diaphorase histochemistry can be used as a means of histologically mapping NOS in the brain (Hope *et al*, 1991; Dawson *et al*, 1991; Bredt *et al*, 1991). The technique is effective despite there being several different types of NADPH-diaphorase present in the brain, not all of which are related to NOS activity; following paraformaldehyde fixation most of the brain's NADPH-diaphorase is inactivated and what remains correlates well with soluble nNOS activity (Matsumoto *et al*, 1993). As an alternative technique, antibody staining and/or *in situ* hybridisation may be used to directly locate NOS, and these techniques have been used with success to validate NADPH-diaphorase staining (Bredt *et al*, 1991a). L-Citrulline, the by-product of NO synthesis has also been found to be colocalised with NADPH-diaphorase (Pasqualotto *et al*, 1991), further supporting the use of NADPH-diaphorase as an indicator of the presence of NO synthase.

I.4.(iv). NITRIC OXIDE IN THE CENTRAL NERVOUS SYSTEM

The first identification of nitric oxide in a physiological role in the central nervous system was by Garthwaite *et al* (1988; 1989), who showed that cerebellar granule cells release nitric oxide following stimulation with NMDA. Subsequently, the neuronal

isoform of NOS was identified (Bredt *et al*, 1990; Bredt and Snyder, 1990) and the ability of NMDA-induced NO release to elevate cyclic GMP concentrations noted (Bredt and Snyder, 1989).

Immunohistochemical staining reveals that the brain isoform of NOS only occurs in neurones (Bredt *et al*, 1991a), although some studies have identified the inducible form of NOS in astrocytes (Simmons and Murphy, 1992; Wallace and Bisland, 1994). NOS positive neurones represent only a small proportion of the total population, with much regional variation, and no clear colocalisation with any one particular conventional neurotransmitter (Bredt *et al*, 1990; Bredt *et al*, 1991a). Neurones which are positive for NOS are significant in a number of respects, not least in their resistance to certain neurodegenerative disorders, including Alzheimer's disease (Hyman *et al*, 1992), Huntington's chorea (Ferrante *et al*, 1985) and stroke (Uemura *et al*, 1990).

One of the first functions to be suggested for neuronal NO was in the formation of hippocampal long-term potentiation (LTP), where it was postulated to act as a retrograde messenger to activate the presynaptic terminals (O'Dell *et al*, 1991; Haley *et al*, 1992). This observation may in fact be due not to the action of nNOS but of the endothelial isoform of NOS which has been identified in pyramidal cells in the hippocampal CA1 region (O'Dell *et al*, 1994; Dinerman *et al*, 1994).

NO has also been implicated as having a modulatory influence on the action of numerous neurotransmitter systems, including acetylcholine (see for example Ohkuma *et al*, 1995; Prast *et al*, 1995), dopamine (see for example Hanbauer *et al*, 1992), noradrenaline (see for example Stout and Woodward, 1995) and GABA (see for example Zarri *et al*, 1994). The precise mechanism by which these modulations of transmitter release take place are only just becoming clear, with evidence emerging that NO can

influence the fusion of synaptic vesicles with cell membranes and hence the release of transmitter (Meffert *et al*, 1996).

In addition, endothelially derived NO has been observed to have more generalised roles in the brain, including the regulation of cerebral blood flow and the response to hypercapnia (Kovách *et al*, 1992; Iadecola *et al*, 1994). Neuronal NO has been implicated in the adjustment of cerebral blood flow to take into account increased neuronal activity (Irikura *et al*, 1994).

I.4.(iv).a. *Targets for nitric oxide*

Nitric oxide is well known as an activator of soluble guanylate cyclase, but the resultant action of cyclic GMP is less well established. Nitric oxide also has several other identified actions with relevance to the central nervous system; the following paragraphs outline the possible next steps following NO synthesis.

I.4.(iv).a.1. Soluble guanylate cyclase

Soluble guanylate cyclase (i.e. that which can be stimulated by NO) is one of a family of cyclases. It is a dimer, each subunit carrying both a catalytic domain and a haem binding domain, although both subunits are necessary for the enzyme to be functional (Buechler *et al*, 1991; Nakane and Murad, 1994). NO appears to activate soluble guanylate cyclase by interaction with the haem moiety (Murad, 1994) although the enzyme also possesses thiol groups which could be capable of modification by NO or other redox agents (Brandwein *et al*, 1981; Kamisaki *et al*, 1986).

Following its synthesis by soluble guanylate cyclase, cGMP has many possible target molecules. There are at least two cGMP-dependent protein kinases, GKI and

GKII which may in turn have complex actions of their own, including ion channel regulation (Lincoln and Cornwell, 1993). GKII has been shown to increase its phosphorylation in response to application of NO, while both GKI and GKII have been found to be widely distributed in the CNS, including high concentrations in the brainstem, thalamus and cortex (El Husseini *et al*, 1996). Cyclic GMP can also interact with certain types of mammalian CNS ion channels directly, including the activation of non-selective cation channels in retinal ganglion cells (Ahmad *et al*, 1994) and photoreceptors (Kurenny *et al*, 1994), and olfactory epithelia (Lincoln and Cornwell, 1993). Cyclic GMP can also activate a cationic current in thalamocortical neurones (Pape and Mager, 1992); this is discussed in detail in section 4.(viii).

Increased concentrations of cGMP can modulate other second messenger systems, including a negative effect on production of inositol 1,4,5-trisphosphate (Hirata and Murad, 1994). The exact mechanism of this effect and the nature of the particular molecule with which cGMP interacts to cause this effect remain to be established.

I.4.(iv).a.2. Direct interactions of nitric oxide with ion channels

Nitric oxide has an effect on a Ca^{2+} current in retinal photoreceptors, such that the current's activation properties are altered; this is independent of cGMP production (in contrast to the cGMP mediated effects on photoreceptors discussed above) (Kurenny *et al*, 1994). Cyclic-GMP-independent effects of NO on Ca^{2+}-dependent K^+ currents outside the CNS have been described, in vascular smooth muscle (Bolotina *et al*, 1994) and gastric fundus (Kitamura *et al*, 1993).

Nitric oxide has been suggested to negatively modulate NMDA receptor function (Manzoni *et al*, 1992b; Manzoni and Bockaert, 1993; Fagni *et al*, 1995) with no effect

on non-NMDA ionotropic excitatory amino acid receptors [although there has been a report that NO-donors can increase the affinity of AMPA receptors for their ligand, Dev and Morris (1994)]. It has been suggested (Lei *et al*, 1992) that NO could have an action at the redox modulatory site of the NMDA receptor to inhibit the receptor's function, but other studies have failed to confirm this view (Hoyt *et al*, 1992). It has been suggested (Fagni *et al*, 1995) that NO may require divalent cations with which to form `clusters', which may then inhibit the channel. However, in an *in vivo* preparation, unlike those from which the hypothesis was drawn, a cGMP mediated effect may override any direct effect of NO. If it is the case that NO negatively modulates NMDA receptors there are two significant implications: firstly, NO could exert a negative feedback effect to switch off its own production and secondly, NO may have a neuroprotective role [see section 4.(vi)].

I.4.(v). EXPERIMENTAL INTERVENTION INTO CNS NITRIC OXIDE SYSTEMS

I.4.(v).a. *Inhibition of nitric oxide synthase*

Several competitive inhibitors of nitric oxide synthase are commercially available. The older and more extensively used of these drugs (N^G-nitro-L-arginine [L-NNA], N^G-monomethyl-L-arginine [L-NMMA] and L-arginine methylester [L-NAME]) are analogues of L-arginine, the substrate for NOS, with substitutions at the guanidino nitrogen; the D-isomers are ineffective. They are inhibitors at all isoforms of NOS, although they differ in their relative selectivities (Reif and McCreedy, 1995). When investigating nNOS *in vivo*, therefore, these compounds share the problem of uncertainty as to the degree of unwanted inhibition of endothelial NOS in the cerebral vasculature which may mask the results. More recently developed compounds, not based upon L-

arginine, are thought to be more useful in studies of NO synthesis by nNOS; these include 7-nitroindazole (7-NI) (Babbedge *et al*, 1993; Moore *et al*, 1993; MacKenzie *et al*, 1994). 7-NI appears to be selective for nNOS by virtue of a difference in its uptake between endothelial cells and neurones, rather than by a difference in its selectivity for eNOS versus nNOS *per se*, as it potently inhibits eNOS in homogenated tissue (Wolff *et al*, 1994). Recently, a sodium salt of 7-NI (7-NINa) has been developed (Silva *et al*, 1995), which because of its enhanced solubility should expand the range of possible experimental uses for this NOS inhibitor. Another recently discovered NOS inhibitor, 1-(2-trifluoromethylphenyl)imidazole (TRIM) is selective for nNOS over eNOS both *in vivo* and *in vitro* (unlike 7-NI) (Handy *et al*, 1996a; Handy *et al*, 1996b); its selectivity for nNOS has been suggested to be caused by an interaction with nNOS's tetrahydrobiopterin binding site (Handy and Moore, 1997).

I.4.(v).b. *L-arginine*

The rate limiting factor in the synthesis of NO is the availability of its precursor L-arginine (Palmer *et al*, 1988b). An excess of L-arginine can therefore be used to artificially elevate concentrations of NO; this has been achieved by Do *et al* (1994) using iontophoretic application. The non-active isomer, D-arginine, can be used as an effective control in such experiments.

I.4.(v).c. *Nitric oxide donor compounds*

NO-donors are compounds which contain an NO molecule within their structure and which can release it under certain circumstances. Some of these compounds have been used therapeutically for many years: the nitrovasodilators glyceryl trinitrate and

amyl nitrite have well established effects on the cardiovascular system which can be used to treat conditions such as angina pectoris. It is only recently, however, that the mechanism of action of such drugs has been elucidated (Needleman *et al*, 1973; Needleman and Johnson, 1973; Kimura *et al*, 1975; Diamond and Blisard, 1976; DeRubertis and Craven, 1976; Schultz *et al*, 1977; Kukovetz *et al*, 1979; Gruetter *et al*, 1980; Ignarro *et al*, 1981). As the importance of the L-arginine/nitric oxide system became apparent, experimenters began to employ compounds related to the nitrovasodilators as a means of delivering molecules of NO to target cells without the technical difficulty involved in using NO as a gas.

One of the most extensively used of these compounds is sodium nitroprusside (SNP), which has been used in many studies as an NO-donor, both in the periphery (Schultz and Schultz, 1977) and in the CNS (Cui *et al*, 1994). SNP decomposes spontaneously to release NO and sodium ferrocyanide in a process which is accelerated by light (Feelisch and Noack, 1987). Limitations of this compound have become apparent, however, especially in the CNS; East *et al* (1991) reported an inhibition of NMDA receptor-mediated responses following application of SNP which could not be attributed to NO, since the effects could not be replicated using alternative NO-donor compounds while similar observations followed application of the chemically related but non-NO producing compound ferricyanide. Other studies (Manzoni *et al*, 1992a; Kiedrowski *et al*, 1992; Oh and McCaslin, 1995) have reached a similar conclusion using different preparations. Thus, SNP provides a problem in the interpretation of data, since in systems involving NMDA receptors it cannot be known whether its effect is due to NO production or to the effect of the by-product ferrocyanide ions. Similar problems are inherent in the use of all NO-donors; the effects of the by-products of NO production

must be taken into account by performing suitable control experiments.

Other NO-donors have been used without encountering problems as severe as those of SNP. Some of these compounds are discussed below:

SIN-1: The derivative of molsidomine, 3-morpholinosydnonimine (SIN-1) (Nishikawa *et al*, 1982), appears to be among the most successful NO-donors, as its breakdown product SIN-1C is biologically inactive (Manzoni *et al*, 1992b). SIN-1 is relatively stable in solution, being able to continuously release NO for some 6-8 hours (Hess *et al*, 1993); Manzoni *et al* (1992b) found that the half maximal rate of NO release from SIN-1 occurred 4 hours after production of the solution.

S-nitrosothiols: These compounds, which include S-nitrosocysteine (SNOC), S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (SNOG or GSNO), have the general structure of R-S-NO, and are being increasingly used as NO-donors, while SNOC has been postulated to be an endogenous NO carrier or sink (Myers *et al*, 1990). In a study of platelet aggregation, GSNO was found to be more effective than either SNAP or SIN-1, having both a higher potency and a long half-life, but with a rate of decomposition which was increased in the presence of glutathione or ascorbate (Park, 1988; Radomski *et al*, 1992). These properties make GSNO the donor of choice for many experiments, provided the possible effects of breakdown into glutathione can be eliminated by performing adequate controls. S-Nitrosothiols have their breakdown (and hence their NO release) accelerated by the presence of transition metal ions (Singh *et al*, 1996).

Iron complexes: Complexes such as Roussin's Black Salt have been infrequently used as NO generating compounds (Flitney *et al*, 1992).

Miscellaneous: (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (FK-409) is a structurally novel NO-generating compound which has recently been observed to have an accelerated rate of breakdown in the presence of sulphydryl compounds such as cysteine or glutathione (Kita *et al*, 1994; Fukuyama *et al*, 1996). Its novel character means this compound has so far been used little as an experimental NO-donor, but it has been suggested to have clinical applications.

Caged nitric oxide compounds have been used by some workers (Pou *et al*, 1994; Makings and Tsien, 1994); they involve a stable complex of NO which can be released upon irradiation.

NONOates (1-substituted diazen-1-ium-1,2-diolates, compounds containing the $[N(O)NO]^-$ group, for example spermine-NO, whose polyamine group precludes its use in the CNS because of possible effects at NMDA receptors) are formed by exposure of a nucleophilic species to NO gas in the absence of oxygen. The result is a stable compound (in its solid form) which will release NO in solution (Hrabie *et al*, 1993).

I.4.(v).d. *Electrochemical detection of NO*

A method for the direct electrochemical measurement of NO was described by Shibuki and Okada (1991), using a gas-permeable capillary tube electrode. This technique is useful for the measurement of low concentrations of NO over short periods of time. An alternative was proposed (Malinski and Taha, 1992; Malinski *et al*, 1993), using a carbon-fibre electrode assembly, which can measure NO over a greater range of

concentrations, but which is less useful *in vivo* due to the possible interference of a number of other biological molecules with the detection of NO.

I.4.(v).e. *Interactions with the guanylate cyclase/cyclic GMP system*

As described above, one of the possible modes of action of NO is via stimulation of the cytosolic enzyme guanylate cyclase to elevate the intracellular concentration of the cyclic nucleotide cyclic guanosine monophosphate (cGMP); this system is vulnerable to pharmacological manipulation in several ways.

Garthwaite *et al* (1995) have described the action of 1H-[1,2,4]-oxadiazolo-[4,3,a]-quinoxalin-1-one (ODQ), a novel and selective inhibitor of soluble guanylate cyclase. This compound has subsequently been used to demonstrate that NO-dependent hippocampal long-term potentiation is mediated via the guanylate cyclase/cGMP system, as it can be blocked by application of ODQ (Boulton *et al*, 1995). ODQ can also be used to reveal cases in which the action of NO-donors is mediated via cGMP (Southam *et al*, 1996; Fedele *et al*, 1996); in coming years it should prove to be a highly useful tool in the elucidation of the means by which nitric oxide exerts its effects. Soluble guanylate cyclase can also be inhibited by methylene blue (Martin *et al*, 1985), although this drug is thought to have complications at other sites in the NO pathway (Mayer *et al*, 1993).

The effects of increased levels of cGMP can be mimicked by a cGMP analogue, 8-bromo-cGMP (8-Br-cGMP) (Vocci *et al*, 1978), which is membrane-permeable and so acts in a manner similar to cGMP itself. 8-Br-cGMP is especially useful when used in conjunction with ODQ and NO-donors: in such cases, if NO is acting through guanylate cyclase and cGMP the effects of the donor should be blocked by ODQ but those of 8-Br-cGMP should not.

The intracellular level of cGMP can also be increased by the inhibition of its breakdown using phosphodiesterase inhibitors. cGMP is hydrolysed to 5'-GMP by a family of phosphodiesterase enzymes. These enzymes are differentiated from each other according to their relative abilities to hydrolyse cGMP or cAMP; although all members of the family can to some extent breakdown both cGMP and cAMP, some degree of selectivity for one of the cyclic nucleotides can be observed (Sonnenberg *et al*, 1993; Sonnenberg and Beavo, 1994). Similarly, the enzymes differ in their susceptibility to inhibitors: isobutylmethylxanthine (IBMX) is a non-selective inhibitor of phosphodiesterase enzymes, while zaprinast (M&B22948) appears to exhibit selectivity towards type V (relatively cGMP-selective) phosphodiesterases (Gillespie and Beavo, 1989). These drugs have been employed to particularly useful effect in the boosting of cGMP levels in readiness for its immunocytochemical localisation (de Vente and Steinbusch, 1992; de Vente *et al*, 1995; de Vente *et al*, 1996).

I.4.(v).f. *Miscellaneous*

Several other methods for the intervention into nitric oxide's physiology and pharmacology have been described. NO gas in solution can be used directly as an alternative to NO-donors, although this presents technical difficulties; conversely, haemoglobin has frequently been used for its ability to scavenge NO and so lower its concentration. Recently, a strain of mouse lacking the gene for neuronal NO synthase has been developed; these mice show behavioural abnormalities including aggressive and hypersexual tendencies (Nelson *et al*, 1995) and show an increased resistance to NMDA induced excitotoxic lesions (Ayata *et al*, 1996), but such observations should not be over-interpreted, as the lack of nNOS may have influenced the development of a number

of other neurotransmitter systems.

The various means of experimental intervention into the nitric oxide/cGMP system are summarised in Figure 4.

FIGURE 4: *Summary of the means of experimental manipulation of the nitric oxide/cGMP system*

(overleaf)

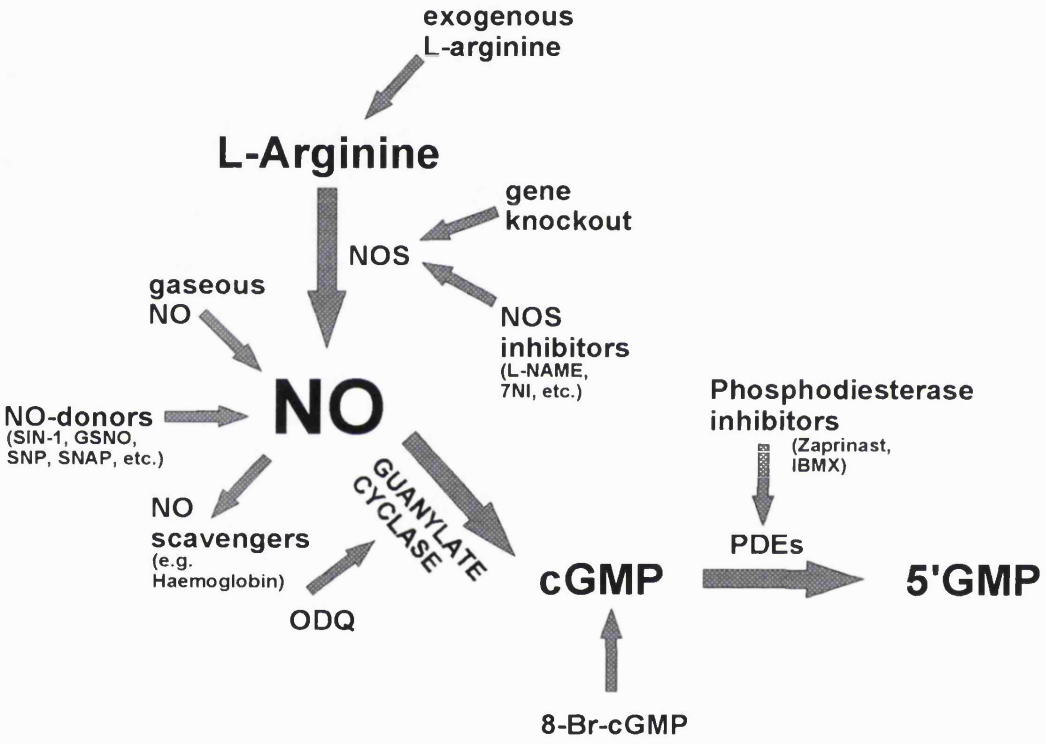

exogenous
L-arginine
L-Arginine
gene
knockout
NOS
gaseous
NO
NOS
inhibitors
(L-NAME,
7NI, etc.)
NO-donors
(SIN-1, GSNO,
SNP, SNAP, etc.)
NO
Phosphodiesterase
inhibitors
(Zaprinast,
IBMX)
NO
scavengers
(e.g.
Haemoglobin)
GUANYLATE
CYCLASE
PDEs
cGMP
5'GMP
ODQ
8-Br-cGMP

FIGURE 4

Summary of the means of experimental manipulation of the nitric oxide/cGMP system

This figure contains a representation of the pathway for the synthesis and principal effect of nitric oxide. Names on large arrows represent enzymes. See section I.4.(v).

I.4.(vi). NITRIC OXIDE IN NEUROTOXICITY AND NEUROPATHOLOGY

It is well established that excess activation of NMDA receptors and the increase in intracellular Ca^{2+} concentration which follows can be responsible for causing neuronal injury (Choi, 1988). Since neuronal NOS is dependent on the Ca^{2+} concentration for it activation, Dawson *et al* (1991; 1993) tested the involvement of NOS in NMDA neurotoxicity and found that such neuronal injury could be attenuated by the presence of NOS inhibitors or haemoglobin, implicating NO in this process. Conversely, NO-donors have been shown to be neurotoxic (Dawson *et al*, 1993). Transgenic mice lacking the gene for nNOS are also resistant to NMDA-induced neurotoxicity relative to the wild-type (Ayata *et al*, 1996). The toxicity of NO following NMDA stimulation appears not to be mediated by NO itself but rather by peroxynitrite ($ONOO^-$), the reaction product of NO and superoxide (O_2^-) (Radi *et al*, 1991a; 1991b), as the presence of superoxide dismutase (which catalyses the conversion of superoxide to hydrogen peroxide) is protective against NMDA and NO-donor toxicity (Dawson *et al*, 1993). Guanylate cyclase and cGMP are not involved in NO-mediated neurotoxicity, since the permeant analogue of cGMP, 8-Br-cGMP, does not exhibit any toxic effects (Lustig *et al*, 1992; Dawson *et al*, 1993).

Some workers have found grounds for controversy regarding the role of NO in neurotoxicity, following the observation in some preparations of a lack of effect or even a neuroprotective role for NO (Demerle-Pallardy *et al*, 1991; Manzoni *et al*, 1992b; Regan *et al*, 1993); this can be accounted for in a number of ways. Firstly, as discussed in a review article by Dawson and Dawson (1996), the exact circumstances of neuronal culture are critical to whether sufficient NO can be produced to mediate neurotoxicity. Secondly, as has been proposed by Lipton *et al* (1993), the alternative redox forms of

NO (nitrosonium, NO^+ and nitroxyl, NO^-) may be neuroprotective while NO itself is neurodestructive through its reaction with superoxide ions.

NO and peroxynitrite mediated neurotoxicity are involved in the damage caused by cerebrovascular infarction; increased levels of NO have been measured during ischaemic conditions (Malinski *et al*, 1993). Studies which prevent peroxynitrite formation by the over-expression of superoxide dismutase have been shown to reduce the size of the lesion following focal ischaemia (Kinouchi *et al*, 1991). Studies using the neuronal NOS-selective NOS inhibitor 7-nitroindazole have shown that this agent is effective in reducing infarct volume, thus implying that the NO which causes the neuronal damage is of neuronal, rather than endothelial, origin (Dalkara *et al*, 1994).

I.4.(vii). NITRIC OXIDE IN THALAMIC SENSORY NEUROTRANSMISSION

There are several lines of evidence, both anatomical and physiological, to suggest a role for nitric oxide in the modulation of sensory neurotransmission in the thalamus.

Anatomical evidence for the ability of neuronal terminals in the thalamus to release nitric oxide comes form the identification of neurones containing NOS; these neurones are cholinergic, have their cell bodies in the peribrachial and laterodorsal tegmental nuclei of the brainstem and have been identified using a combination of NADPH-diaphorase histochemistry and staining for choline acetyltransferase, a marker for cholinergic neurones (Vincent and Kimura, 1992; Bickford *et al*, 1993), and by using *in situ* hybridisation (Sugaya and McKinney, 1994). Noradrenergic and serotonergic neurones in the brainstem which also innervate the thalamus do not stain positively for NADPH-diaphorase. The function of the cholinergic input to the thalamus from the brainstem is thought to be the modulation of the mode of firing of thalamocortical

neurones and possibly in the determination of the level of arousal [Sillito *et al*, 1983; Eysel *et al*, 1986; McCormick and Prince, 1987; McCormick and Pape, 1988; Hu *et al*, 1989; Steriade *et al*, 1990; Kayama *et al*, 1992; Lu *et al*, 1993, and section I.1.(iv).].

The first physiological identification of a role for NO in thalamic neurotransmission was the demonstration *in vitro* by Pape and Mager (1992), using pressure pulse ejection of the NO-donors SIN-1 and SNP, that NO shifts the voltage-dependence of the thalamocortical relay cells' hyperpolarisation activated cation current I_h [see section I.1.(iii)] in a positive direction. This effect was mimicked by 8-Br-cGMP, suggesting that NO's effect has the guanylate cyclase/cGMP system as an intermediate. The outcome of this influence on I_h is to move the membrane potential into the range which favours tonic firing, and so to lessen the probability of the cell entering burst mode. This result suggests the possibility that NO and acetylcholine can act in combination to control the firing mode of thalamocortical neurones, with NO affecting I_h and acetylcholine affecting an outward K^+ current (Jahnsen and Llinás, 1984a, 1984b) with the same net effect in each case.

Do *et al* (1994), using the technique of push-pull perfusion in the rat ventrobasal thalamus, demonstrated that the precursor of NO, L-arginine, is released into the extracellular environment upon somatosensory stimulation. Given that L-arginine in the CNS is predominantly localised in glial cells (Aoki *et al*, 1991), Do and co-workers speculated that their observation of L-arginine release may represent a transfer between cellular compartments, i.e. from the site of storage (glial cells) to the site of NO synthesis (cholinergic terminals). This postulation has gained some support from the work of Grima *et al* (1996), which shows a transfer of L-arginine from glia to cortical neurones in culture. The study of Do *et al* (1994) went on to reveal that iontophoretic application

of L-arginine to cells in the ventrobasal thalamus *in vivo* causes a potentiation of sensory responses and responses to the excitatory amino acid agonists NMDA and AMPA which could be blocked by the NOS inhibitor L-NAME.

Using a similar rationale, other workers have iontophoretically applied NOS inhibitors to cells in the dLGN of the anaesthetised cat (Cudeiro *et al*, 1994a; 1994b). These studies revealed that NOS inhibitors reduce the magnitude of visual responses, but unlike Do *et al* (1994), suggest that NO's effect is mediated selectively by NMDA receptors. Cudeiro *et al* (1996) also showed that NO-donors enhance visual responses in dLGN. Cudeiro *et al* (1994b) failed to find an effect of 8-Br-cGMP in dLGN, but this may be due to technical problems, which will be discussed in section D.2.(iii).

The most direct physiological evidence for NO in the thalamus comes from the recent work of Miyazaki *et al* (1996) who used a carbon fibre electrode for the direct measurement of NO (or at least of its metabolites). They found that the NO concentration increases upon stimulation of the laterodorsal tegmental nucleus of the brainstem, one of the origins of the cholinergic neuronal input to the thalamus and the putative site for NOS activity. These results are consistent with the idea that NO is released from cholinergic terminals in the thalamus upon the excitation of these neurones; a lower basal release of NO was also detected.

Taken in combination, the various studies outlined above point to the ability of NO to be both synthesised and released within thalamic nuclei. Since the capability of NO to influence thalamocortical relay cells has also been demonstrated, it is safe to suggest that NO has an important physiological action in sensory neurotransmission through thalamic nuclei and may be able to influence the integrity of information reaching the cortex.

SECTION I.5

AIMS OF THE PRESENT RESEARCH

The extensive evidence for the presence of nitric oxide in thalamic sensory relay nuclei has been detailed in the preceding section (for example Bickford *et al*, 1993), along with the evidence for an excitatory action of nitric oxide on thalamocortical relay cells (Pape and Mager, 1992; Cudeiro *et al* 1994a; 1994b; 1996; Do *et al*, 1994). Bearing in mind the evidence from these studies, a strategy was devised to test the following hypotheses:

(i) nitric oxide donors when iontophoretically applied should have an excitatory action on VB or dLGN neurones.

(ii) chemically dissimilar NO-donors should produce similar effects if their action is mediated by NO.

(iii) nitric oxide synthase inhibitors should conversely be inhibitory when applied to VB or dLGN neurones.

(iv) the breakdown products of NO-donor compounds should not replicate the effects of the donor itself (e.g. glutathione should not mimic the effects of GSNO).

(v) L-arginine when applied to dLGN cells should have a facilitatory action, as has been previously observed in VB.

(vi) if thalamic nitric oxide exerts its action via the guanylate cyclase/cyclic GMP system, it should be possible to replicate the effects of increasing or decreasing the NO concentration by pharmacologically manipulating the level of cyclic GMP in relay neurones.

To test these hypotheses, an *in vivo* rat preparation was used [with methodology as detailed by Salt (1987)]. This technique combines single cell extracellular neuronal recording with iontophoretic drug application; in this way, the effects of drugs on both physiological and pharmacological stimuli can be determined.

It was decided to test the various compounds under investigation in both VB and dLGN; the rationale behind this decision was to test whether the difference between the two nuclei (i.e. the presence of inhibitory interneurones in dLGN) would have any influence over the nitric oxide system, and whether the modulatory actions of the nitric oxide system are comparable in somatosensory and visual thalamic nuclei.

CHAPTER 2

METHODS

M.1. THE PRINCIPLES OF IONTOPHORESIS

M.1.(i). INTRODUCTION

The term iontophoresis describes an electrophysiological experimental technique used for the administration of drugs to a localised area by passing an electrical current through the drug solution. It was first used by Nastuk (1953) and del Castillo and Katz (1955) to deliver acetylcholine to the neuromuscular junction. The following subsections will attempt to give an overview of the applications, advantages and limitations of the technique, along with a discussion of its relevance to the present study.

M.1.(ii). BASIC PRINCIPLES

Iontophoretic drug administration to a single neurone or small group of neurones requires an experimental setup in which a micropipette of suitable dimensions (i.e. with a tip diameter of 5μm or less) is filled with an ionised drug solution and connected via a wire (usually silver or platinum) to a device for the precise generation of small electrical currents, in the nanoamp range, such as the "Neurophore" (Medical Systems Corporation). Using such an arrangement, the source of current can be used to pass an ionic current of either negative or positive polarity through the drug solution, carrying ions of the drug out of the micropipette tip and onto the cells being studied. For instance, a negative current would cause the ejection of negatively charged ions from the micropipette.

In addition, when drug ejection is not required, the same arrangement can be used

to apply a continuous low current of the opposite polarity to prevent leakage of the drug out of the micropipette by diffusion. This current is referred to as a retaining current.

As discussed above, iontophoresis is a process which requires ionisation of the compound to be ejected. Despite this, however, by a process known as electro-osmosis, non-ionised compounds can be ejected. Electro-osmosis arises from aqueous solutions because of the formation of a layer of negative charge close to the surface of the glass, leaving the remainder of the solution relatively positive. Passing a positive current therefore carries fluid containing the test compound out of the barrel tip.

M.1.(iii). IONTOPHORESIS MICROPIPETTES

Micropipettes for iontophoresis usually take the form of an assembly of borosilicate glass capillary tubes which are fused together before being heated and pulled to a tip. In this way it is possible to create a combination of a central microelectrode for the purpose of neuronal extracellular recording with a number of outer barrels to contain the drugs which are under investigation.

Following the pulling process it is necessary to break the sealed tip of the electrode/micropipette assembly to the required diameter; this is necessary to allow both recording and the passage of ions through the tip. The electrode tip is pushed against a glass sphere or other suitable surface while being observed under a microscope. This process is referred to as "bumping back" the electrode tip, and for an electrode assembly consisting of a central recording barrel and six outer barrels should result in a tip diameter of approximately 5μm.

Following the process of electrode filling described below, it is possible to use the Neurophore or similar apparatus to check the electrical resistance of each individual

electrode barrel; this should typically be in the range 10-40MΩ. A greater barrel resistance than this may require the tip to be bumped back to a larger diameter or a replacement electrode to be chosen. A lower barrel resistance may risk leakage of the drug from the electrode tip.

At all stages of the electrode manufacturing process it is imperative that the capillary tubes be kept free of dust or other particles. If small solid particles should find their way to the inside of the tip of the electrode and the lumen becomes blocked, current will be unable to pass and the electrode assembly becomes unusable.

M.1.(iv). DRUG SOLUTIONS

For iontophoresis to be successful in the conventional way, the drugs which are to be used must satisfy several criteria.

Firstly, drugs must be sufficiently soluble in water or saline to enable their placement in the micropipette. Compounds which are only sparingly soluble in water may present problems if they are precipitated from the solution as solid particles will tend to increase the electrical resistance of the electrode barrels and ultimately block the passage of ionic current. Compounds which are only soluble in organic solvents such as ethanol or dimethyl sulphoxide (DMSO) create other problems in that control experiments must be carried out to eliminate the contribution of the vehicle to the results.

In addition to being sufficiently soluble, compounds for iontophoresis must also be sufficiently polar to be able to carry ionic current. However, for certain compounds, notably amino acids, the experimenter is able to artificially manipulate the polarity of the drug under investigation by raising or lowering the pH of the solution to add or dissociate protons to or from the drug molecules. This is achieved by simply adding a small quantity of sodium

hydroxide or hydrochloric acid to the drug solution (for example, amino acids such as NMDA are ejected at pH 8). For other compounds, there is less effect of pH on the extent of ionisation, but addition of sodium hydroxide can increase the rate of ejection of the test compound be the carriage of its molecules in the water of hydration of sodium ions.

M.1.(v). CURRENT BALANCING

To compensate for the artefactual responses which may be observed from neurones due to the effects of current, it is usual to fill one of the outer barrels with 1M sodium chloride . This can then be used to automatically balance the sum of all ejection currents (under the control of the Neurophore system or its equivalent) by carrying a current of equal magnitude but of opposite polarity. In this way, therefore, it is possible to reduce the magnitude of changes in the firing rate of cells which may occur in response to application of electrical current. Current balancing is not a flawless solution to the problem of current artefacts, however, as it can cause artefactual effects of its own.

For instance, the ejection of sodium ions from the current balancing barrel may have important effects on certain neurones. Also, there is an at least hypothetical problem in which the balance barrel's current interferes with current from other barrels; it is conceivable that in a situation where agonist responses are being tested against a second compound, the change in the magnitude of balance current at the onset of the second compound's ejection would alter the properties of the agonist ejection.

M.1.(vi). EJECTION OF MARKER DYES FOR HISTOLOGY

Iontophoretic ejection in addition to being a highly useful means of drug delivery is also a method by which histological markers or tracers can be inserted into neuronal

tissue. These include dyes for the location of the electrode tip, released extracellularly, such as the pontamine sky blue used in the present study, as well as intracellular tracers such as horseradish peroxidase.

M.1.(vii). THE RATIONALE BEHIND THE USE OF IONTOPHORESIS TO STUDY THE ROLE OF NITRIC OXIDE IN THE THALAMUS

For the present study is was necessary to choose a methodology which could deliver drugs accurately to a precise location in very small quantities. Delivering, for instance, a nitric oxide donor compound to an *in vivo* preparation by iontophoresis has the advantage over *in vitro* studies in which drugs are applied to the bathing solution that it influences only the immediate locality of the neurone being studied (although iontophoretic studies can also be performed *in vitro*). In addition, using this method it is possible to limit the time period of the drug's application to a very brief period; it is potentially of greater physiological relevance, therefore, preventing any toxic effects of nitric oxide which may occur following prolonged application. These factors, it was thought, outweighed the possible disadvantage of the nitric oxide donor compounds decomposing in the electrode before they were required to do so. Working *in vivo* ensures the presence of a complete thalamic circuit, including brainstem modulatory influences, which may be crucial to the function of nitric oxide in this system. It also allows the use of physiological stimuli, i.e. vibrissal or visual stimulation.

M.2. EXPERIMENTAL PROCEDURES

M.2.(i). ELECTRODE MANUFACTURE

Seven-barrelled iontophoresis electrodes were constructed in a multi-stage process from filamented 100mm long glass capillary tubes with an internal diameter of 1.17mm and an outer diameter of 1.5mm (obtained from Clark Electromedical, no. GC150 TF-10).

Three such capillary tubes were cut in half and the six resultant pieces positioned in pin-vices to surround a fourth, full-length tube. This assembly was then heated in a gas flame to enable the seven-tube arrangement to be twisted together to fuse the barrels and pulled by hand to an apex. Upon removal from the pin-vices, the upper end of the outer barrels was gently heated and pulled slightly away from the central long barrel to enable easier insertion of a tube for the filling of the finished electrode.

Following this stage, the electrode tip was pulled gravitationally using an electrically heated coil, held in micromanipulator. The temperature of the coil could be regulated by adjusting the current. The electrode was again mounted in a pin-vice and held vertically with the coil surrounding the tip; the coil was heated and a weight attached to the tip end of the electrode (via a short piece of capillary tube and a further pin-vice). The tip if the electrode was stretched to the required length (in excess of 6mm) by heating the coil and moving it in a downward direction. The final stage involved shaping the profile of the electrode tip by further heating of the coil and allowing the weight to drop by gravity, forming a point with an even taper and a tip diameter of approximately 1μm, which was inspected under a microscope. Electrodes with unsatisfactory tip profiles were rejected and repulled. Completed electrodes were stored in jars so as to maintain them in a dust-free state.

Prior to commencement of each experiment, the tip of an electrode was broken under a microscope by pushing it against a glass sphere, to enable the passage of current and the ejection of drugs. This process resulted in a tip diameter of approximately 5μm.

The central barrel of each electrode was filled with a 4M aqueous solution of sodium chloride; this barrel was used for extracellular single cell recording via silver wire. One outer barrel was filled with a 1M sodium chloride solution for automatic balancing of iontophoretic currents. A further barrel was filled with 2.5% pontamine sky blue dye (BDH) in 0.5M sodium acetate and 0.5M sodium chloride solution, for use as a marker of the site of recording.

M.2.(ii). ANIMAL MAINTENANCE AND SURGERY

Wistar rats were used in somatosensory studies; Lister Hooded rats were used in visual experiments to avoid any problem which may arise due to the absence of normal visual pigments in the albino Wistar strain. Adult male rats were used in all cases; animals were maintained with food and water *ad libitum*. All procedures were carried out within the regulations stipulated by the Animals (Scientific Procedures) Act (1986).

Anaesthesia was initiated by placing the animal in a sealed atmosphere containing halothane. Maintenance anaesthesia was under urethane at 1.2g/kg by intraperitoneal injection. Supplementary doses were given whenever necessary. Lignocaine hydrochloride solution (2% w/v plus adrenaline) was injected subcutaneously at the site of all incisions, and lignocaine gel applied to the tips of the animal's ear bars.

Following induction of anaesthesia, a tracheostomy was performed, a glass cannula inserted into the trachea and the animal mounted in a stereotaxic frame with the vertical zero plane through the lambda suture and the inter-aural line. An electrically heated

thermostatically controlled blanket maintained the animal at 37°C throughout the experiment. The electrocardiogram was continuously monitored on an oscilloscope and by an audio beep; the electroencephalogram was monitored on an oscilloscope via screws inserted into the skull which were held in place and insulated with dental acrylic. It was decided not to monitor the animals' blood pressure in order to minimise surgery; this was though not to constitute a disadvantage, as none of the test drugs were being administered systemically.

A hole of 5mm diameter was drilled in the skull above the right thalamus and the dural membrane carefully removed. The electrode position was located relative to the lambda suture and according to the stereotaxic coordinates of Albe-Fessard *et al* (1971). A vertical zero point was established by touching the electrode down on the cortical surface; the electrode was then advanced by an electronically controlled stepping micromanipulator (Digitimer). Warm agar solution (3% in 0.9% sodium chloride solution) (Sigma) was placed around the electrode so as to cover completely the craniotomy hole and protect the cortex from the effects of drying.

M.2.(iii). VARIATIONS OF METHODOLOGY FOR dLGN EXPERIMENTS

In order to effectively provide visual stimuli in experiments in which the lateral geniculate nucleus was to be the site of recording, it was necessary to mount the animal in a head-holder device, attached to one side of the stereotaxic frame, to facilitate the removal of the mouth-bar and left-hand side of the stereotaxic frame; as a result, the animal's left field of vision was unobstructed, and visual stimulation could be achieved more successfully. The head-holder consisted of a U-shaped metal plate which was screwed to the skull such that the craniotomy lay in the middle and the lambda suture was not

obscured. This assembly was further secured by liberal application of dental acrylic.

To prevent corneal drying, a drop of light mineral oil was placed in the animal's left eye. The pupil was dilated to 3-4mm diameter by topical application of a small drop of atropine methyl nitrate (40%, Sigma) in 0.9% sodium chloride solution; this compound is less-lipid soluble and therefore less likely to enter the CNS than atropine sulphate. A suture above the eye was tied to the head-holder to maintain the eye in an open state.

M.2.(iv). RECORDING AND IONTOPHORESIS

Iontophoretic drug ejection was controlled by a Neurophore system (Medical Systems Corporation) which enables sequential ejections of drugs as part of a computer controlled stimulation cycle. Ejection currents were automatically balanced; retaining currents of 5-25nA of the correct polarity (i.e. opposite to the ejection polarity) were applied to hold drugs within the electrode barrels prior to ejection. The impedance of each electrode barrel was tested at the beginning of an electrode penetration and accepted as satisfactory if less than 20MΩ. Details of drug solutions used for iontophoresis can be found in a later section.

M.2.(v). HISTOLOGY

For the purpose of verification of the site of recording, pontamine sky blue dye (2.5% in 0.5M sodium acetate and 0.5M sodium chloride solution) was ejected from the electrode at the end of some experiments; ejection with a current of 150μA for 10 minutes resulted in a spot of dye in the brain tissue which could easily be observed following histological procedures.

The brain was carefully removed and fixed in paraformaldehyde (4% in phosphate

buffered saline), prior to being cut on dry ice into 60μm sections. Sections were mounted onto glass microscope slides coated in 3% gelatin solution, and stained to reveal Nissl substance with 1% Neutral Red solution, according to a standard procedure. Glass coverslips were applied to the stained slides with DePeEx mounting medium. After being allowed to dry, sections were viewed under a microscope; the stereotaxic atlas of Albe-Fessard *et al* (1971) was used to identify the site of the dye-spot.

M.2.(vi). DRUG SOLUTIONS FOR IONTOPHORESIS

To be suitable for iontophoretic ejection, a drug must satisfy certain criteria: it must be sufficiently soluble in aqueous solution and also be polar to a great enough extent to carry current. The polarity of most drugs was enhanced by careful adjustment of the pH. In all cases, great care was taken to avoid contamination with particles such as dust which may block iontophoresis electrodes. Drugs used for these experiments are listed below with their concentrations, pH and any other relevant details.

N-methyl-D-aspartate (NMDA): 50mM aqueous solution, pH 8, adjusted with 1M sodium hydroxide solution; as used by Do *et al* (1994). Obtained from Tocris Cookson.

(R,S)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA): 100mM aqueous solution, pH 8, adjusted with NaOH; as used by Do *et al* (1994). Obtained from Tocris Cookson.

Sodium nitroprusside (SNP): 10mM aqueous solution, pH 8, adjusted with NaOH. Made freshly on the morning of each experiment. Nitroprusside anion ejected with

negative current. Obtained from Sigma.

Carbachol: A non-selective cholinergic agonist. For early experiments a 200mM aqueous solution was used, which proved to be difficult to retain in the electrode. Subsequently the concentration was reduced to 100mM and later to 50mM to correct the problem. Regardless of concentration, the solution was adjusted to pH 4 with 1M hydrochloric acid; solution formed from carbachol chloride, and therefore ejected as a positive ion. Obtained from Sigma.

S-nitrosoglutathione (GSNO): 10mM aqueous solution, pH 8, adjusted with NaOH. Made freshly for each experiment. Contains carboxyl groups, therefore forms a negative ion in a basic solution and was ejected with negative current. A gift from Dr. K-Q. Do.

Reduced glutathione (GSH) and oxidised glutathione (GSSG): both forms of glutathione were used in a 10mM aqueous solution, pH 8, adjusted with NaOH. Prepared and ejected as for GSNO, to provide an effective control. Made freshly for each experiment. Gifts from Dr. K-Q. Do.

3-morpholinosydnonimine (SIN-1): 40mM aqueous solution, pH 4.5, adjusted with HCl. Made freshly for each experiment from the chloride, therefore ejected as a positive ion.. Obtained from Tocris Cookson.

8-bromoguanosine-3,5-cyclic monophosphate (8-Br-cGMP): 50mM aqueous solution, pH 8, adjusted with NaOH. Made freshly for each experiment. Preparation based on that

of Hentall (1995). Obtained from Sigma.

L-arginine and D-arginine: 50mM aqueous solution in both cases, pH 4, unadjusted (as used by Do *et al,* 1994). Ejected as a positive ion, due to -NH groups in acidic solution. Obtained from Sigma.

Nitro-L-arginine (NNA): 50mM aqueous solution, pH 4, adjusted with HCl. Ejected as a positive ion, in the same way as L-arginine, whose structure it incorporates. Obtained from Sigma.

N(G)-nitro-L-arginine methylester (L-NAME): 50mM aqueous solution, pH 4, adjusted with HCl. Ejected as a positive ion in the same way as L-arginine, whose structure it incorporates.. Obtained from Sigma.

M.2.(vii). PHYSIOLOGICAL STIMULATION

M.2.(vii).a. *Somatosensory stimulation*

When recording from the ventral posteromedial nucleus of the thalamus, neurones respond to movement of a single facial vibrissa or to deflection of the hairs on a small area of hairy skin on contralateral side of the face. For these experiments, stimulation was achieved by means of pulses of air directed at the receptive field. Pulses were electronically gated (by a Digitimer D4030system) to be of a precise duration (10ms or 1000ms) and to occur at specific moments in time; the area stimulated was restricted by passing the pulse through a hypodermic needle. The air-jet could therefore be directed at a small area of rat's face by manually adjusting its position with a micromanipulator. Two durations of air-jet

were used in order to be able to resolve any difference in the effects of test compounds on non-NMDA receptor mediated responses (short duration) and NMDA receptor mediated responses (prolonged duration) (Salt, 1986; Salt, 1987). A stimulus cycle (total duration 6000ms) consisting of a 10ms air-jet followed 4000ms later by a 1000ms air-jet was repeated 5 times, and subsequently cumulated.

M.2.(vii).b. *Visual stimulation*

For experiments in which the electrode was recording from the lateral geniculate nucleus, physiological stimulation was achieved by means of a green light emitting diode placed in the receptive field. Initially, the topography of the receptive field was mapped using a hand-held light source and then the LED, held by a micromanipulator, was placed in to the receptive field's centre, 1-5cm from the eye, and the position adjusted to give the best response from the neurone. The LED was flashed for 500ms at the beginning of a 10 second cycle, which was repeated 10 times and subsequently cumulated.

M.2.(viii). ELECTRONIC APPARATUS

The recording from the central barrel of the electrode was conducted via silver wire to a headstage preamplifier, before being passed to an Axoprobe 1A microelectrode amplifier (Axon Instruments). The signal was transferred to a "Neurolog" system (Digitimer), in which it was filtered, and gated to produce pulses in response to the firing of a single neurone. The non-gated signal was displayed on one channel of an oscilloscope (Tektronix 5111A), while the gated spikes were displayed on the other channel in an expanded timebase and in storage mode. This arrangement allowed the gating of spikes to be manually adjusted so as to ensure that action potentials from only one cell were counted.

Pulses generated in response to action potentials were than collected by a personal computer, being transferred via a Cambridge Electronic Design 1401 interface.

M.2.(ix). DATA ANALYSIS AND STATISTICAL TESTING

Following collection by personal computer, pulses were then processed off-line to produce peristimulus time histograms (PSTHs) which represent patterns of neuronal firing with time as the abscissa. The PSTHs could then be integrated to produce counts of neuronal firing during stimulation; in this manner, control responses and responses elicited during ejection of a test compound could be compared.

The magnitude of a drug's effect was computed by calculating the test response as a percentage of the control response (where control was defined as the values from the cycle immediately preceding the onset of the ejection of the test compound); over a number of cells, this figure was then used to calculate the mean percentage of the control. Where histograms were to be plotted, the standard error of the mean was also calculated.

Following observation of any consistent trend in the results, data were statistically tested using a non-parametric technique; the test used in all cases was the Wilcoxon Signed Rank test. This non-parametric test was chosen to be applicable to non-normally distributed paired data. Probabilities of less than 0.05 were considered to be significant.

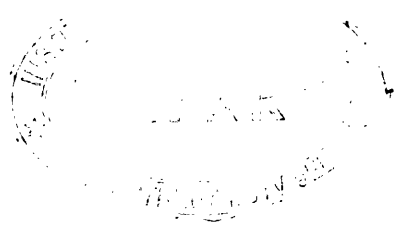


FIGURE 5: *Methods summary*

(overleaf)

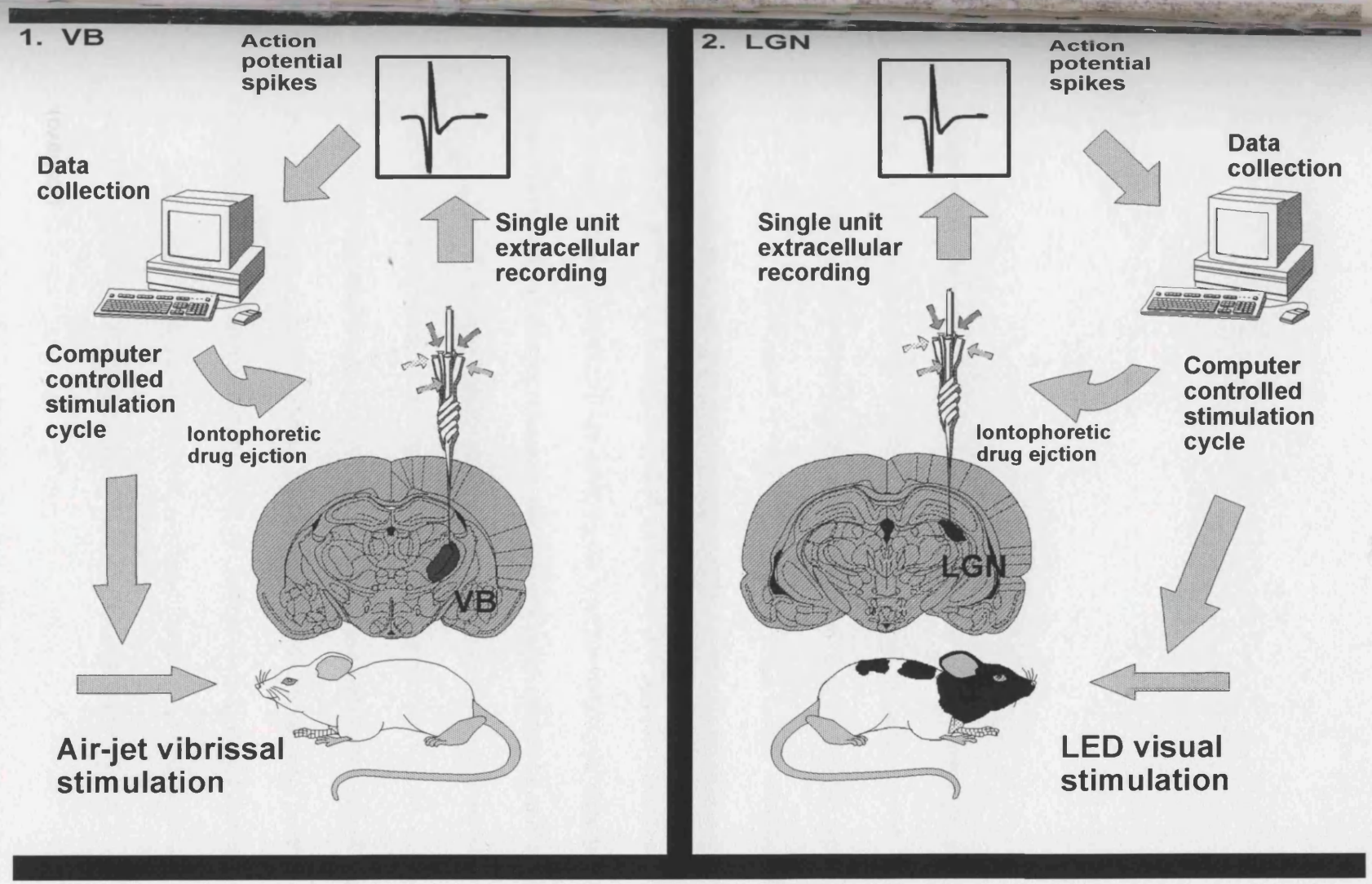

1. VB
Action potential spikes
Data collection
Single unit extracellular recording
Computer controlled stimulation cycle
Iontophoretic drug ejction
VB
Air-jet vibrissal stimulation
2. LGN
Action potential spikes
Data collection
Single unit extracellular recording
Computer controlled stimulation cycle
Iontophoretic drug ejction
LGN
LED visual stimulation

FIGURE 5

Methods summary

This figure represents the sequential arrangement of the apparatus.

The left-hand panel shows the arrangement used for VB experiments, employing a Wistar rat and air-jet stimulation. Action potential spike recordings were collected by a personal computer, which was also responsible for the control of a cycle of iontophoretic drug ejection. The highlighted area of the coronal section of rat brain indicates the ventrobasal complex, comprising the ventral posterolateral and ventral posteromedial subdivisions.

For dLGN experiments, a similar arrangement was used, as shown in the right-hand panel. In this case, however, the rats used were of the pigmented Lister Hooded strain and physiological stimulation was by means of a flashed LED. The highlighted area of the brain section indicates the dLGN.

CHAPTER 3

RESULTS

R.1. RECEPTIVE FIELDS AND RECORDING LOCATION

For all experiments, stereotaxic coordinates were derived from the stereotaxic atlas of Albe-Fessard *et al* (1971). The location of electrode penetrations used for VB experiments were 2.9mm lateral and 5.2mm rostral of the lambda suture and for dLGN experiments were 3.3mm lateral and 3.6mm rostral of lambda. VB neurones were encountered at between 4.2mm and 5.8mm from the cortical surface, while dLGN neurones were found at depths of between 3.0mm and 4.5mm.

Single unit extracellular recordings were made from a total of 56 ventrobasal thalamus neurones and 43 dLGN neurones. Receptive fields of VB cells were single vibrissae or small areas of hairy skin on the face which responded in a consistent manner to manual deflection or air jet stimulation; dLGN neurones were found to respond to short duration stationary flashes of light. These observations were consistent with the correct localisation of the electrode tip in either VB or dLGN, and in cases where histological analysis was performed, the dye-spot was found to be in the appropriate thalamic nucleus. Experiments accounting for 32 of the VB neurones and all dLGN experiments were dye-spotted.

In the light of technical problems in early cases of histological analysis in which the dye-spot was lost, presumably having been washed out during the staining process, the methodology was revised to ensure a larger and more resilient dye-spot. This was successfully achieved by ejecting dye from the electrode with a current of higher amplitude (300 μA) and for a longer duration (15 minutes).

R.2. SODIUM NITROPRUSSIDE

Sodium nitroprusside (SNP) has been a very widely used nitric oxide donor (see section I.4.(v).c.). It is sufficiently soluble and sufficiently polar to be suitable for use in iontophoresis, easily forming a clear pale yellow solution at 10mM and pH 8 in water. To minimise any problem with degradation of the compound over time, the solution was prepared freshly for each experiment immediately prior to the filling of each electrode. SNP is susceptible to photodegradation, and for this reason the electrode was protected from exposure to light between filling and its insertion into the brain.

SNP was applied to 8 VB neurones, and produced an effect at relatively small iontophoretic currents: -5nA was used in all cases, with a retaining current of 15nA. An effect was achieved at 4 - 8 minutes of ejection at this current magnitude. Responses to NMDA were selectively and significantly ($p<0.05$, Wilcoxon Signed Rank Test) inhibited in the presence of SNP, being reduced to $23 \pm 6\%$ of control (mean $\pm$ standard error of mean). Responses to 10ms air-jet stimuli and 1000ms air-jet stimuli were both similarly reduced, to 60 ± 12 % of control ($p<0.05$) and $60 \pm 8\%$ of control ($p<0.05$), respectively. Responses to AMPA and carbachol were unaffected by SNP at this current, being reduced to $92 \pm 9.8\%$ and $94 \pm 17.0\%$ respectively, both statistically non-significant.

Complete recovery to control levels of agonist responses occurred in only 2 of the 8 cells tested, after allowing a period of at least 30 minutes. During this time, AMPA and carbachol responses remained stable, suggesting that SNP has a prolonged effect.

Figure 6 is a typical VB cell to which SNP was applied; Figure 7 shows mean data for the population of 8 cells to which SNP was applied, which are also shown in tabular form in Table 1.

Following these results (and see section I.4.(v).c.) and results with alternative NO donors it was considered inappropriate to attempt to use SNP as an NO donor for dLGN neurones. For confirmation of this view, however, SNP was applied to one dLGN neurone, and it was found to produce an effect similar to that on VB cells; responses to NMDA and visual stimulation were both reduced, while that to AMPA was unchanged.

FIGURE 6: *Sodium nitroprusside selectively inhibits responses to NMDA and air-jet stimulation*

(overleaf)

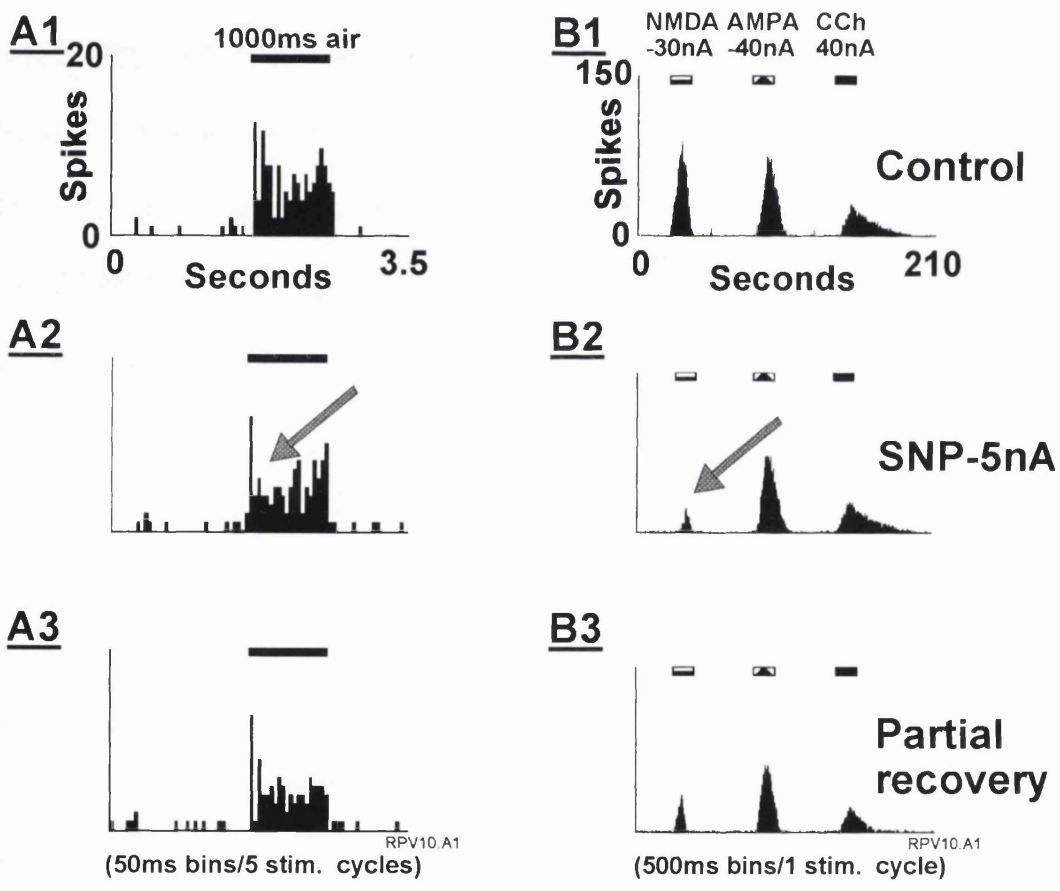

A1
1000ms air
20
Spikes
0
0
Seconds
3.5
B1
NMDA AMPA CCh
-30nA -40nA 40nA
150
Spikes
0
Control
0
Seconds
210
A2
B2
SNP-5nA
A3
B3
Partial
recovery
RPV10.A1
RPV10.A1
(50ms bins/5 stim. cycles)
(500ms bins/1 stim. cycle)

FIGURE 6

Sodium nitroprusside selectively inhibits responses to NMDA and air-jet stimulation

This figure shows peristimulus time histograms (PSTHs) representing action potential spike counts of a single VB neurone counted into bins and displayed with time as the abscissa.

The neurone was recorded in cycles lasting 4 minutes; in each cycle, 5 repeats of air jet stimulation were followed 4 seconds later by 15 second periods of NMDA, AMPA and carbachol.

In column A, the responses of the cell to physiological (air-jet) stimuli are shown, with control data shown in record A1. Each record represents five cycles of stimulation displayed in a cumulative form. The horizontal bar represents the duration of the air-jet. Record A2 shows the effect of SNP applied for 8 minutes with an iontophoretic ejection current of -5nA, while A3 shows the response of the cell after a recovery period of 8 minutes. It can be seen (indicated by the arrow) that SNP caused the cell's response to air-jet stimulation to be reduced from 139 to 116 spikes. Partial recovery had occurred by record A3.

In column B, the responses of the cell to the excitatory amino acid agonists NMDA and AMPA are shown, along with responses to the cholinergic agonist carbachol. Symbols represent the periods of agonist ejection. Records are shown on a timebase different from that used for column A, to maximise clarity, and represent a single cycle of stimulation (i.e. are not cumulated). It can be seen in record B2 that the response to NMDA was selectively inhibited by 8 minutes of SNP ejection at -5nA (from 1423 to 211 spikes), indicated by the arrow. Only partial recovery occurred after 8

minutes (record B3); full recovery failed to happen in 30 minutes of subsequent recording.

FIGURE 7: *Histogram representing mean data: sodium nitroprusside selectively inhibits air-jet and NMDA responses*

(overleaf)

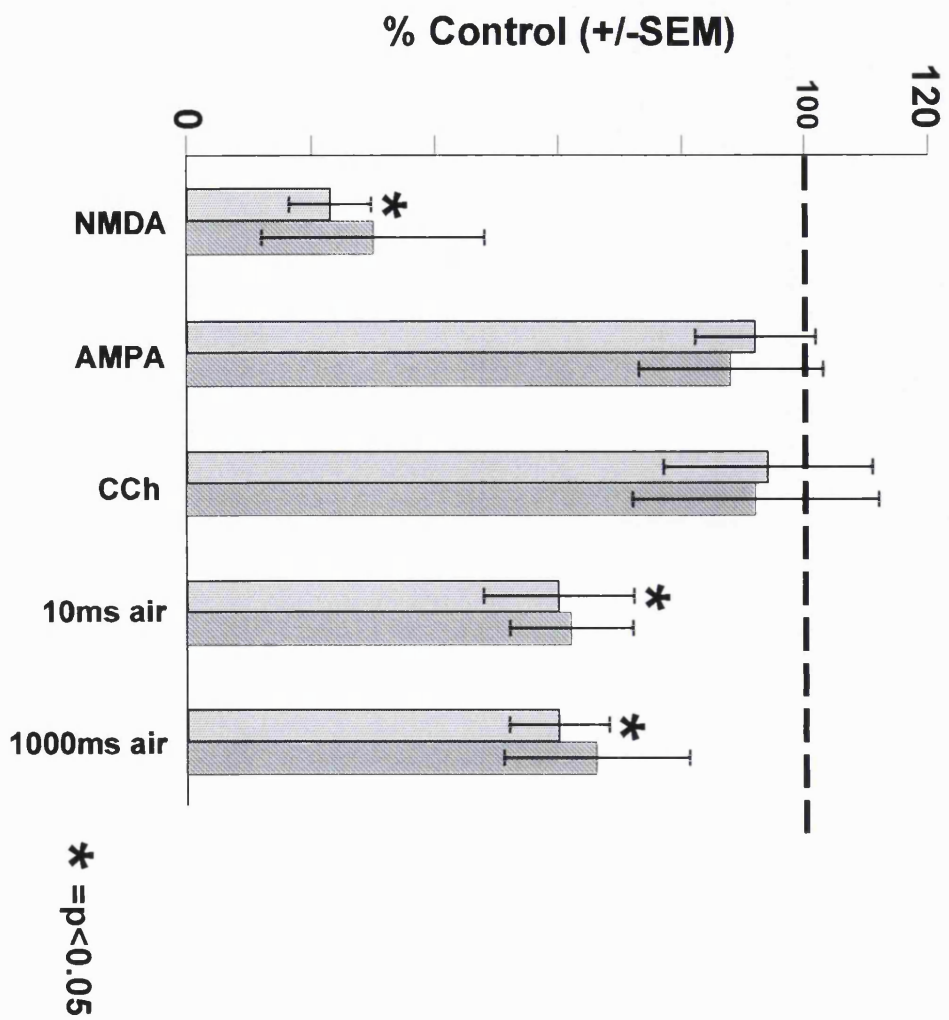

% Control (+/-SEM)
0
100
120
NMDA
AMPA
CCh
10ms air
1000ms air
* =p<0.05

FIGURE 7

Histogram representing mean data: sodium nitroprusside selectively inhibits air-jet and NMDA responses

SNP was applied to 8 VB neurones. The light bars of the histogram represent the mean percentage of control responses during SNP application for the three agonists and air-jet stimuli. Error bars represent plus or minus the standard error of the mean. Asterisks indicate statistical significance versus control ($p<0.05$) according to the outcome of the Wilcoxon Signed Rank test. Dark bars represent response levels after 28 minutes of recovery.

Points to note include the substantial and statistically significant reduction of the responses to NMDA and each of the air-jet stimuli. Responses to AMPA remained unaffected while those to carabchol were variable (note large SEM) but the change was not statistically sigificant. Recovery was poor, possibly indicating a long lasting effect of SNP.

TABLE 1

Summary of the effects of sodium nitroprusside on VB neurones (n=8)

	NMDA	AMPA	Cch	10ms	1000ms
SNP -5nA	23±6.6*	92±9.8	94±17.0	60±12.2*	60±8.1*

(mean % control ± standard error of mean)

* =p<0.05, Wilcoxon Signed Rank test

R.3. 3-MORPHOLINOSYDNONIMINE (SIN-1)

R.3.(i). SIN-1: INTRODUCTION

As discussed in Section I.4.(v).c., SIN-1 is a nitric oxide donor whose breakdown product is biologically inactive, making it particularly suitable for studies of this type. For the present experiments it was prepared at 40mM and pH 4.5 in water, thus being ejected iontophoretically as a positive ion (see methods section M.2.(vi).). Due to the possible problem of breakdown, the solution was made as late as possible and only used for 8 hours.

R.3.(ii). SIN-1: VENTROBASAL THALAMIC NEURONES

SIN-1 was applied to 6 VB neurones with iontophoretic current in the range 25 to 50nA. None of the other compounds under investigation had been applied to these cells. An effect was observed following 4 - 8 minutes of drug ejection.

Responses to NMDA and AMPA were both significantly potentiated by SIN-1 coapplication, the means being increased to $166 \pm 26\%$ and $176 \pm 33\%$ of control values respectively ($p<0.05$ in both cases, Wilcoxon Signed rank test).

Responses to air-jet vibrissal stimulation were also enhanced by ejection of SIN-1. The response to a 10ms air-jet was increased to $203 \pm 34\%$ of control ($p<0.05$) and that to a 1000ms air-jet was increased to $136 \pm 15\%$ of control ($p<0.05$).

Figure 8 shows responses of a typical and representative VB neurone to which SIN-1 was applied; mean data for the population of cells to which SIN-1 was applied are shown graphically in Figure 9 and also in Table 2.

FIGURE 8: *SIN-1 potentiates somatosensory and EAA responses of a typical VB neurone*

(overleaf)

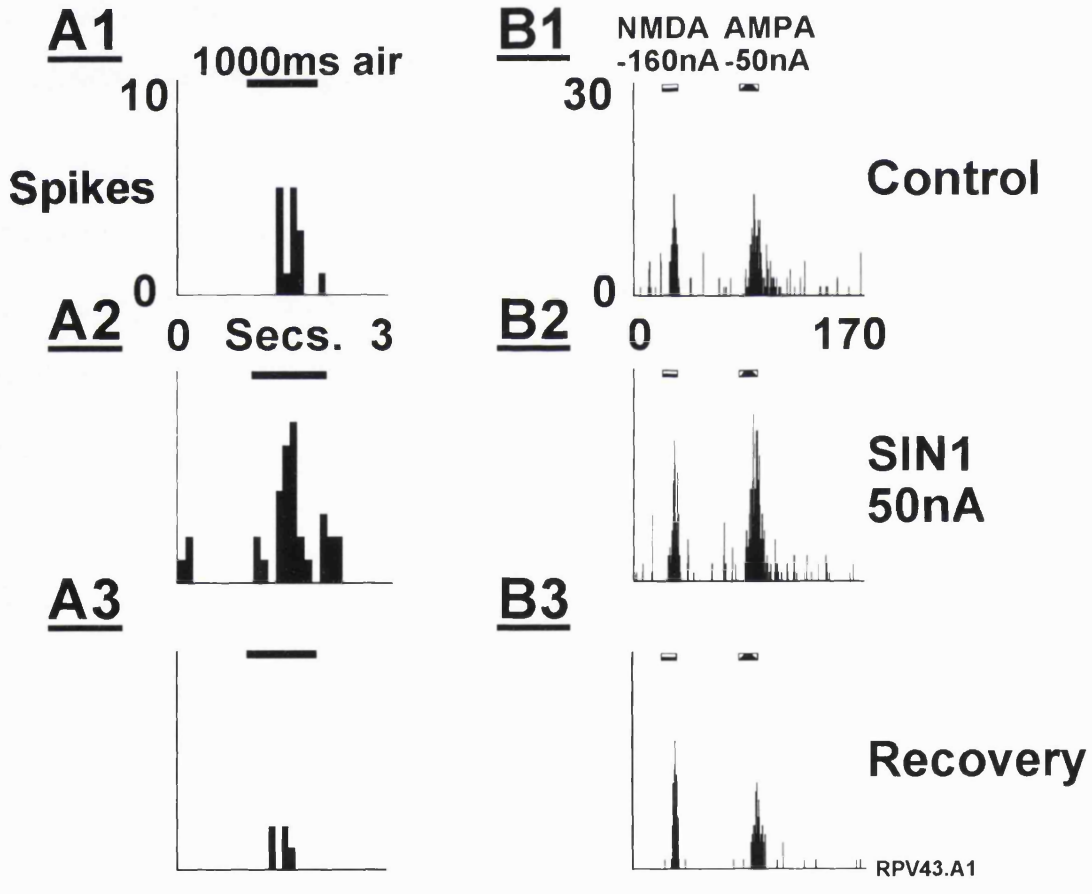

A1
1000ms air
10
Spikes
0
A2
0 Secs. 3
A3
B1
NMDA AMPA
-160nA -50nA
30
Control
0
B2
0
170
SIN1
50nA
B3
Recovery
RPV43.A1
(50ms bins/5 stim. cycles)
(500ms bins/1 stim. cycle)

FIGURE 8

SIN-1 potentiates somatosensory and EAA responses of a typical VB neurone

This figure shows PSTHs representing action potential firing of a single VB neurone plotted against time.

Column A contains record of the cell's responses to air-jet vibrissal stimulation; each record contains five cumulated stimuli. The bar represents the period of the stimulus in each case. Record A2 shows data in the presence of SIN-1 (following 8 minutes of ejection), with the response clearly enhanced (30 spikes) when compared to the control data shown in record A1 (15 spikes). Recovery from the effects of SIN-1 is shown in record A3, 4 minutes after the cessation of ejection.

On a different timebase (and with a single cycle of stimulation in each case), column B shows the effects of SIN-1 on the same cell's responses to the excitatory amino acid agonists NMDA and AMPA. Symbols represent the periods of agonist ejection. In this case the response to NMDA was increased immediately after the onset of SIN-1 ejection (record B2) relative to control (from 95 to 138 spikes), as was the response to AMPA (from 186 to 303 spikes). The effects of 400 seconds recovery from the effects of SIN-1 are shown in record B3.

FIGURE 9: *Histogram representing mean data: SIN-1 causes an enhancement of responses in VB*

(overleaf)

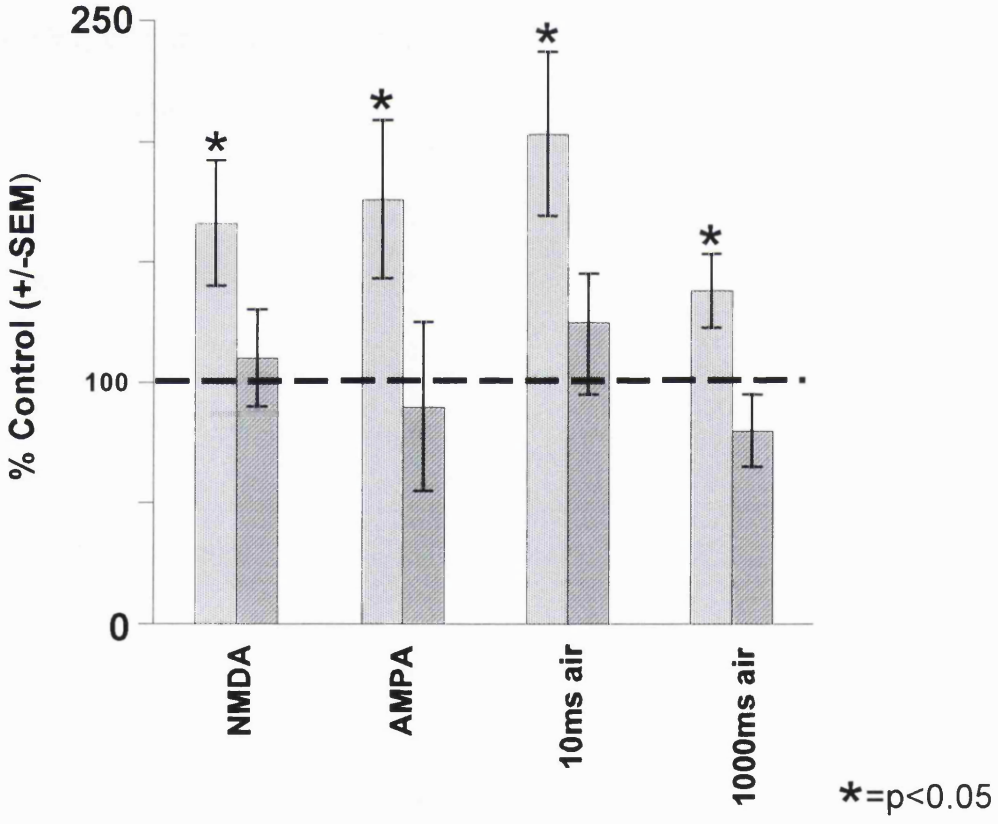

250
100
0
% Control (+/-SEM)
*
*
*
*
NMDA
AMPA
10ms air
1000ms air
*=p<0.05

FIGURE 9

Histogram representing mean data: SIN-1 causes an enhancement of responses in VB

SIN-1 was applied to 6 VB neurones; the histogram shows the mean effects in the form of percentages of control responses (light bars) and the responses following 10 minutes of recovery (dark bars). Error bars represent the standard error of the mean. Asterisks indicate statistical significance, i.e. probabilities of less than 0.05 according to the Wilcoxon Signed Rank test.

It should be noted from this figure that SIN-1 caused an enhancement both of responses of the neurones to excitatory amino acid agonists and responses to air-jet vibrissal stimulation.

TABLE 2

Summary of the effects of SIN-1 on VB neurones (n=6)

	NMDA	AMPA	10ms	1000ms
SIN-1 (25-50nA)	166±26*	176±33*	203±34*	136±15*
(mean % control ± standard error of mean)				

* =$p<0.05$, Wilcoxon Signed Rank test

R.3.(ii). SIN-1: dLGN NEURONES

SIN-1 was applied to 9 dLGN neurones with iontophoretic currents of 10 to 30nA. This population of cells had not received application of any of the other test compounds.

Responses to NMDA were potentiated by SIN-1 to 214 $\pm$ 29% of control values (mean $\pm$ SEM, $p<0.01$); similarly, responses to AMPA were increased by SIN-1 application to 209 $\pm$ 28% of control ($p<0.01$). Visual responses were also potentiated by SIN-1, to 133 $\pm$ 9% of control ($p<0.05$).

Figure 10 shows responses of a typical dLGN neurone to which SIN-1 was applied. Mean data for these 9 cells is shown in Figure 11 and in Table 3.

FIGURE 10: *SIN-1 potentiates visual and EAA responses of a dLGN neurone*

(overleaf)

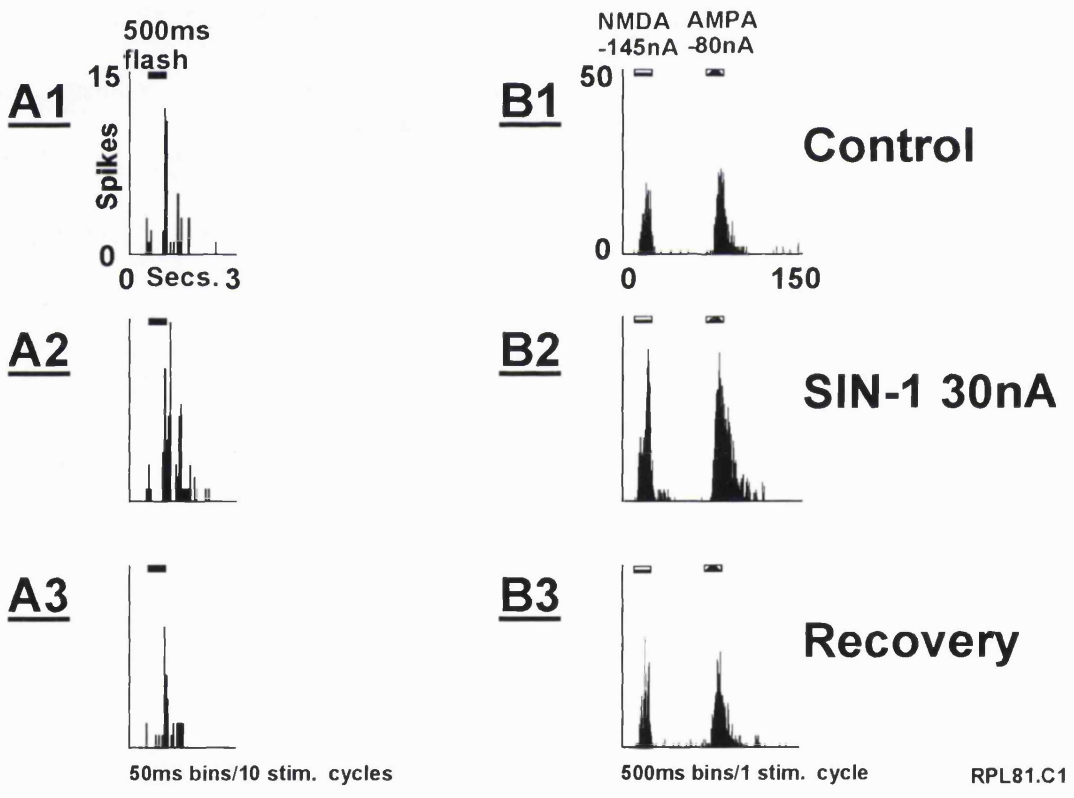

500ms
flash
A1
15
Spikes
0
0 Secs. 3
A2
A3
50ms bins/10 stim. cycles
NMDA AMPA
-145nA -80nA
B1
50
0
0
150
Control
B2
SIN-1 30nA
B3
Recovery
500ms bins/1 stim. cycle
RPL81.C1

FIGURE 10

SIN-1 potentiates visual and EAA responses of a dLGN neurone

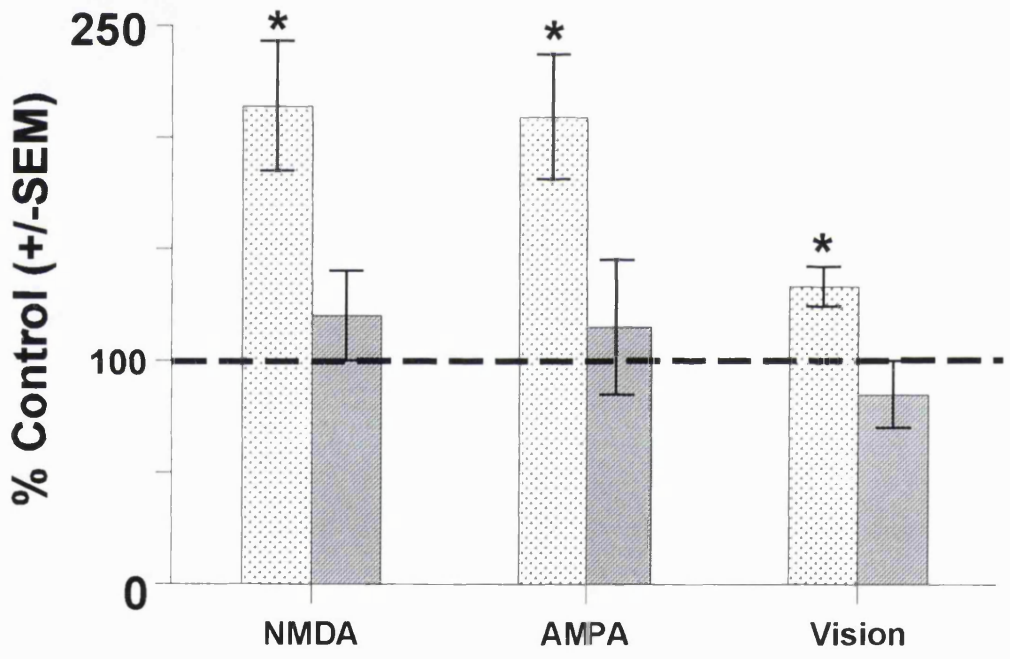
This figure shows PSTHs representing the action potential spike firing a single dLGN neurone collected into bins and plotted against time.

Column A shows records of the cell's responses to a LED flash stimulus; each record contains data from 10 cumulated responses. The horizontal bar represents the duration of the flash stimulus. Record A1 contains control data which can be compared with the response shown in A2, following 4 minutes of SIN-1 application. SIN-1 caused the response to be increased from 43 to 78 spikes. Recovery is shown in record A3.

Column B uses a different timebase and bin size to show responses of the same cell to NMDA and AMPA. Each record represents a single cycle of stimulation (i.e. is not cumulative); symbols represent the periods of agonist ejection. Record B2 reveals that SIN-1 (after 12 minutes of application) caused an increase in the response of this cell to NMDA, from 253 to 536 spikes, and in the response to AMPA, from 434 to 963 spikes. The effect of 6 minutes recovery from the effects of SIN-1 is shown in record B3.

FIGURE 11: *Histogram representing mean data: SIN-1 enhances visual and EAA responses in dLGN*

(overleaf)



* =p<0.05

FIGURE 11

Histogram representing mean data: SIN-1 enhances visual and EAA responses in dLGN

This histogram contains mean data for all of the 9 dLGN cells to which SIN-1 was applied (light bars) and the effects of 6 minutes recovery (dark bars). Error bars represent the standard error of the mean. Asterisks indicate statistical significance ($p<0.05$, Wilcoxon Signed Rank test).

SIN-1 clearly enhanced both visual and excitatory amino acid responses of this population of neurones.

TABLE 3

Summary of the effects of SIN-1 on dLGN neurones (n=9)

	NMDA	**AMPA**	**Vision**
SIN-1 (10-30nA)	213±29*	209±28*	133±9*
(mean % control ± standard error of mean)			

* =$p<0.05$, Wilcoxon Signed Rank test

R.4. S-NITROSOGLUTATHIONE (GSNO)

R.4.(i). GSNO: INTRODUCTION

Like SIN-1, GSNO (or SNOG) is a nitric oxide donor. Unlike SIN-1, however, GSNO has a biologically active breakdown product, glutathione, and as such must have control experiments performed to isolate possibly nitric oxide mediated effects from those mediated by glutathione. The results of experiments with glutathione will be included in this section where relevant to GSNO, while the effects of glutathione in its own right will be included in section R.6.

GSNO is freely soluble in water and was iontophoresed using a negative current from a solution of 10mM and pH 8 (see methods section M.2.(vi).). As with other NO-donors, it was necessary to use a fresh solution of GSNO for each experiment in order to minimise the effects of spontaneous breakdown of the compound.

R.4.(ii). GSNO: VENTROBASAL THALAMIC NEURONES

GSNO was applied to 12 neurones in VB with iontophoretic currents of -40 to -200nA; none of these cells had been previously exposed to any of the other test compounds. In 9 of these neurones, GSNO caused an increase in the response to NMDA (to 123 $\pm$ 12%, mean percentage of control $\pm$ standard error of the mean) and AMPA (to 123 $\pm$ 10%). Both effects were statistically significant, according to the Wilcoxon Signed Rank test ($p<0.05$). No change was observed, however, in the response to carbachol. The remaining 3 neurones of the sample showed no effect following GSNO application.

All of the same 12 neurones exhibited an increase in their response to air-jet vibrissal stimulation following iontophoretic application of GSNO. The response to a 10ms

air-jet was potentiated by GSNO to 124 $\pm$ 7% of control ($p<0.01$) while that to a 1000ms air-jet stimulus was potentiated to 126 $\pm$ 8% of control ($p<0.01$).

Recovery of the cells from the effects of GSNO proved to be highly variable; only 5 of the 9 cells which showed increases in their EAA responses following GSNO application recovered to control levels within 30 minutes. Similarly, only 7 of the 12 cells which had their physiological responses potentiated recovered to control levels within 30 minutes.

Figure 12 is a typical VB neurone to which GSNO was applied. Figure 13 shows one of 4 cells to which GSNO and glutathione were both applied, to contrast their effects and provide evidence that the action of GSNO is mediated via nitric oxide production. Mean data for the 12 cells is shown in histogram form in Figure 14 and in table form in Table 4.

FIGURE 12: *GSNO potentiates somatosensory and EAA responses of a typical VB neurone*

(overleaf)

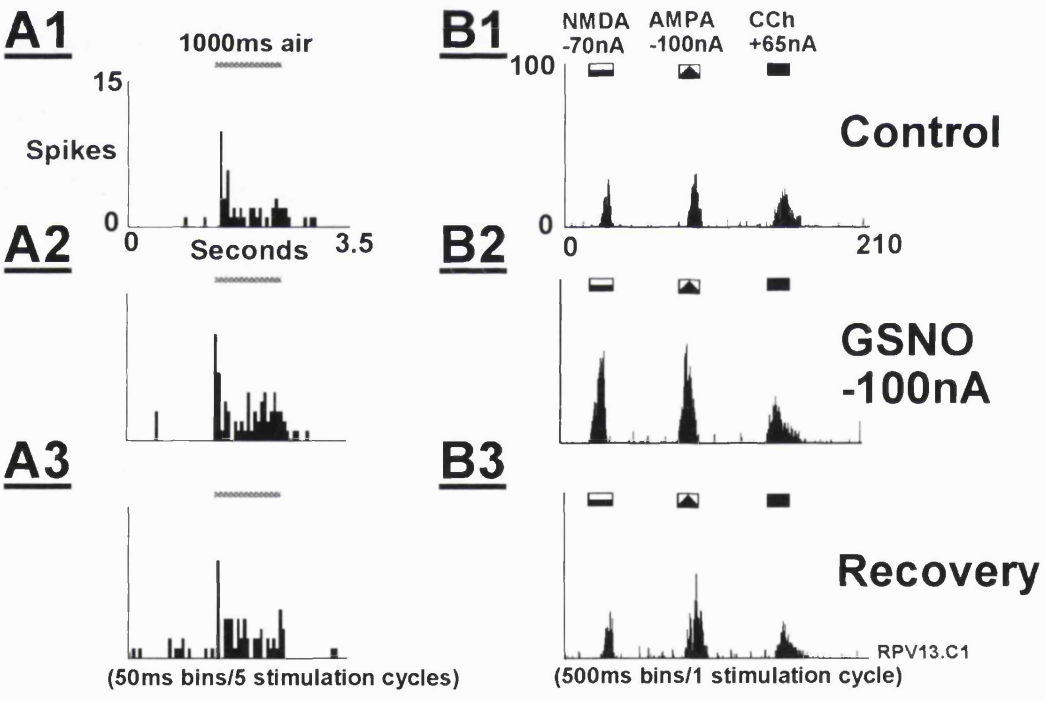

A1
1000ms air
15
Spikes
0
0
Seconds
3.5
A2
A3
(50ms bins/5 stimulation cycles)
B1
NMDA
-70nA
AMPA
-100nA
CCh
+65nA
100
Control
0
0
210
B2
GSNO
-100nA
B3
Recovery
RPV13.C1
(500ms bins/1 stimulation cycle)

FIGURE 12

GSNO potentiates somatosensory and EAA responses of a typical VB neurone

This figure contains PSTHS representing action potential firing of a single VB neurone over time.

Column A contains records of the cell's responses to a 1000ms air-jet stimulus; each record contains cumulative data from 5 cycles of stimulation. The horizontal bars represent the duration of the stimuli. Record A2 (116 spikes) can be contrasted with record A1 (49 spikes) to reveal the effects of 4 minutes of iontophoretic GSNO application.

Similarly column B contains records representing the responses of the same neurone to NMDA, AMPA and carbachol. Symbols indicate the periods of agonist ejection. Each record in this case contains data from a single cycle of stimulation; note that the bin size is greater than in column A for clarity. Record B2 shows that the responses to NMDA and AMPA were increased by 4 minutes of GSNO application to levels greater than the control responses shown in record B1. (The NMDA response was increased from 236 spikes in B1 to 683 spikes in B2; the AMPA response was increased from 356 to 787 spikes). The response to carbachol showed a slight increase (392 to 520 spikes) which was not statistically significant for the population.

Partial recovery of the cell from the effects of GSNO is shown in A3 and B3.

FIGURE 13: *GSNO has an excitatory action on a VB neurone; GSH inhibits responses of the same cell*

(overleaf)

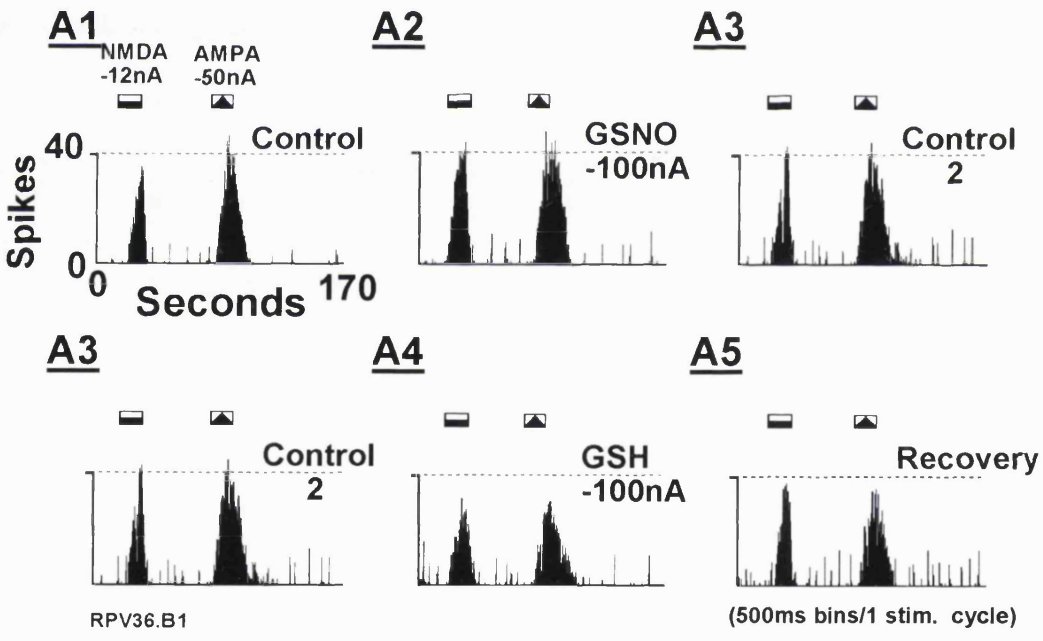

A1
NMDA
-12nA
AMPA
-50nA
Control
40
Spikes
0
0
Seconds
170
A2
GSNO
-100nA
A3
Control
2
A3
Control
2
A4
GSH
-100nA
A5
Recovery
RPV36.B1
(500ms bins/1 stim. cycle)

FIGURE 13

GSNO has an excitatory action on a VB neurone; GSH inhibits responses of the same cell

To contrast the effects of GSNO and glutathione, the two compounds were consecutively applied to the same 4 VB neurones, one of which is shown in this figure. The figure contains PSTHs representing action potential spikes of the cell against time; Symbols represent periods of NMDA or AMPA ejection.

Record A2 shows that GSNO enhanced the cell's responses to NMDA and AMPA (as in Figure 12). After allowing the cell to recover (record A3, shown twice for clarity), GSH was applied at the same iontophoretic current as had previously been used for GSNO. It can be seen from record A4 that the effect was an inhibition of responses to NMDA and AMPA, in contrast to the effects of GSNO.

The contrasting effects of GSNO and GSH are evidence for the action of GSNO being mediated via nitric oxide production, rather than by breakdown into glutathione.

FIGURE 14: *Histogram representing mean data: GSNO enhances sensory and EAA responses in VB*

(overleaf)

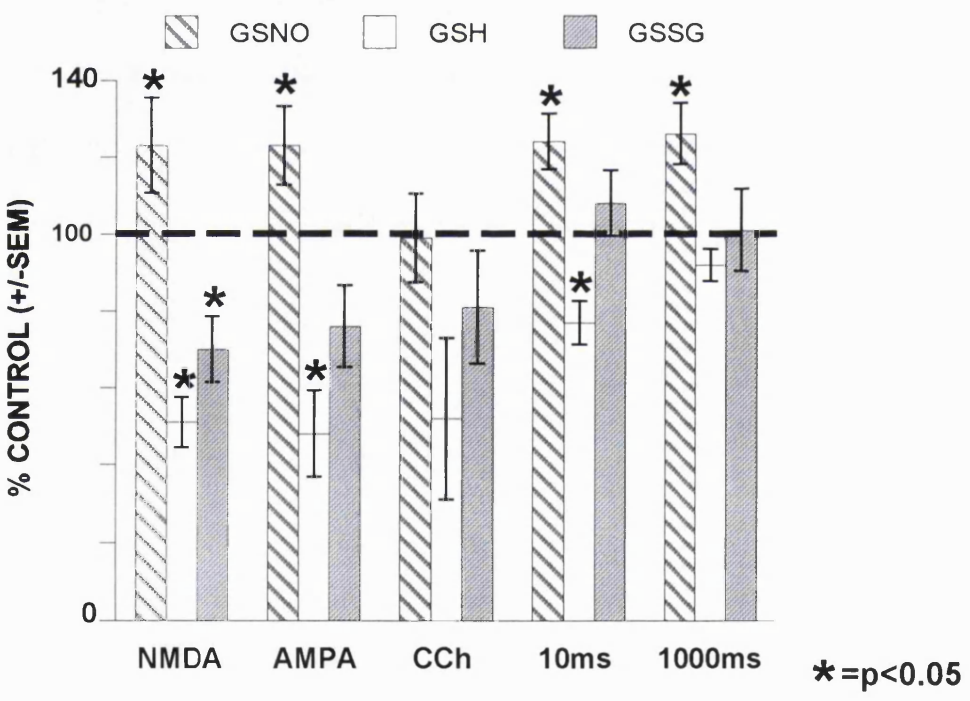

GSNO
GSH
GSSG
140
100
0
% CONTROL (+/-SEM)
NMDA
AMPA
CCh
10ms
1000ms
*=p<0.05

FIGURE 14

Histogram representing mean data: GSNO enhances sensory and EAA responses in VB

This histogram contains mean percentage of control data for the effects of GSNO on VB neurones (n=12), along with the effects of reduced glutathione (GSH, n=6; neurones not those used for GSNO or GSSG) and oxidised glutathione (GSSG, n=10; neurones not those used for GSNO or GSH) for comparison. Error bars represent the standard error of the mean. Asterisks indicate statistical significance ($p<0.05$, Wilcoxon Signed Rank test).

It can be seen that GSNO caused an increase of responses to EAAs and air-jet stimulation to levels greater than control. Glutathione in either form caused either an inhibition of responses or no change, supporting the view that GSNO acts via nitric oxide production rather than through its glutathione moiety. The effects of glutathione will be discussed more comprehensively in the subsequent section.

TABLE 4

Summary of the effects of GSNO on VB neurones (n=12)

	NMDA	AMPA	Cch	10ms	1000ms
GSNO -40 to -200nA	123±12*	123±10*	99±12	124±7*	126±8*

(mean % control ± standard error of mean)

* =$p<0.05$, Wilcoxon Signed Rank test

R.5. NITRIC OXIDE DONORS: SUMMARY

Experiments were performed to apply three different nitric oxide donors to thalamic relay neurones by iontophoresis, the results indicating the usefulness of this technique for investigating nitric oxide systems. As well as illustrating the effects of nitric oxide, the data provided important information regarding the relative merits of the three nitric oxide donors.

SNP was only applied to VB neurones; this was because it produced a result which was unexpected and which was unlike those obtained with the other nitric oxide donors SIN-1 and GSNO. SNP caused a selective inhibition of the cells' responses to NMDA which, it is hypothesised, was due not to nitric oxide production but to a by-product. Support for this hypothesis can be found in the literature (for example East *et al*, 1991). The inhibition of responses mediated by NMDA receptors was also manifested as an inhibition by SNP of responses to air-jet vibrissal stimulation. The effects of SNP are more fully discussed in section D.2.(ii).

SIN-1 is not thought to create any such problems with by-product generation. The present experiments clearly demonstrated a facilitatory action of SIN-1 both on VB neurones and on dLGN neurones. In both nuclei, responses to excitatory amino acid stimuli and sensory stimuli were potentiated during SIN-1 ejection. If SIN-1 does not have other breakdown products with biological actions, it can be interpreted that this effect is mediated by nitric oxide.

GSNO is a nitric oxide donor chemically unrelated to SIN-1. Its profile of effects in VB were identical however, and (as described below) were not mimicked by glutathione, its second breakdown product. Since SIN-1 and GSNO both produced similar results, the suggestion that they are acting via production of nitric oxide is strongly supported.

R.6. GLUTATHIONE

R.6.(i) GLUTATHIONE: INTRODUCTION

The background to the roles of glutathione in the central nervous system is discussed in section I.3. The reasons for the use of glutathione in the present study were twofold. Firstly, as discussed above in Section R.3., it provided a control for the possible effects of GSNO breakdown and thus provided evidence for the action of GSNO being mediated via nitric oxide production. Secondly, glutathione is known to be neuroactive in its own right (see references in Section I.3.) and it was therefore hoped to be able to observe what effects iontophoretic application of glutathione may have on thalamic neurones.

Glutathione in its reduced form (GSH) is a tripeptide and as such can be iontophoresed in essentially the same way as an amino acid; in this case it was prepared as a 10mM aqueous solution at pH 8. The solution was made freshly for each experiment, although this was not to prevent decomposition but merely to facilitate a more direct comparison with GSNO. The oxidised (GSSG) form of glutathione was prepared in the same way.

R.6.(ii). REDUCED GLUTATHIONE (GSH): VENTROBASAL THALAMIC NEURONES

GSH was applied to 6 VB neurones with iontophoretic currents in the range -5 to -100nA. None of these cells had previously been used for any other of the test compounds. A cycle of 4 minutes was established, each cycle consisting of 5 repeats each of the air-jets followed 4 seconds later by ejection of NMDA, then AMPA, then carbachol.

For these 6 cells, the response to NMDA was significantly ($p<0.05$, Wilcoxon Signed Rank test) inhibited by GSH coapplication, to 51 $\pm$ 7% of control (mean percent control $\pm$ standard error of the mean). Similarly, the response to AMPA was inhibited by GSH, to 48 $\pm$ 11% of control. GSH had no statistically significant effect on the response to carbachol.

When applied to the same 6 cells, GSH had an inhibitory effect on the responses to somatosensory (i.e. air-jet vibrissal) stimulation. Responses to a 10ms air-jet were reduced to 77 $\pm$ 6% of control, although the response to an air-jet of 1000ms duration was unaffected being reduced only to 92 $\pm$ 4% of control (not statistically significant).

Figure 15 represents responses of one of the 6 VB cells described above. Figure 16 is a histogram containing mean data for all 6 cells (which is also shown in Figure 14, above), while the same data is shown numerically in Table 5. Figure 13 (above) represents the responses of one of the cells to which received both GSNO and GSH application, in which the effects of the two drugs can be contrasted.

FIGURE 15: *GSH inhibits air-jet and EAA responses of a typical VB neurone*

(overleaf)

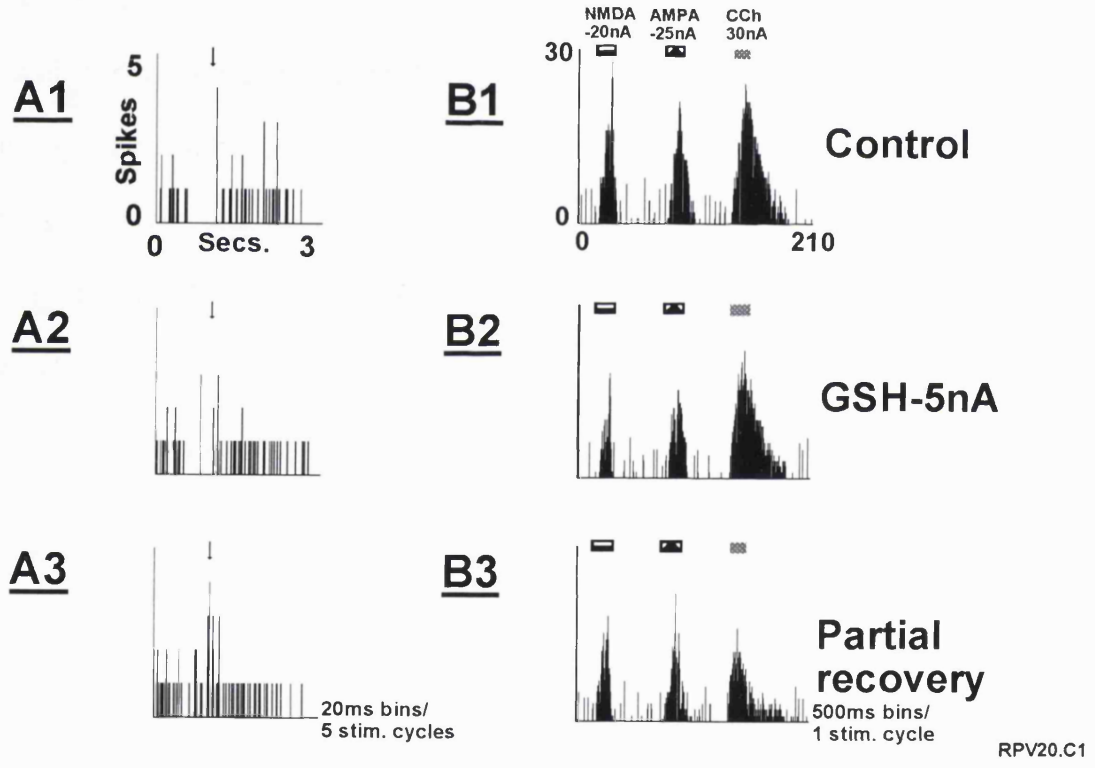

A1
5
Spikes
0
0
Secs.
3
B1
NMDA
-20nA
AMPA
-25nA
CCh
30nA
30
0
0
210
Control
A2
B2
GSH-5nA
A3
B3
20ms bins/
5 stim. cycles
Partial
recovery
500ms bins/
1 stim. cycle
RPV20.C1

FIGURE 15

GSH inhibits air-jet and EAA responses of a typical VB neurone

Figure 15 shows PSTHs representing action potential spike firing of a single VB neurone collected into bins and plotted against time.

Column A contains records of the cell's responses to 10ms air jet stimulation; each record contains 5 cumulative cycles of stimulation. The arrow represent the onset of the stimuli. Record A2 shows data following 16 minutes of GSH application, and it can be seen that the air-jet response was reduced from 17 spikes (control, record A1) to 13 spikes.

With a greater bin size, column B shows responses of the same cell to the excitatory amino acid agonists NMDA and AMPA and the cholinergic agonist carbachol. Symbols represent periods of agonist ejection. From record B2 it can be seen that responses to NMDA were inhibited in the presence of GSH (from 474 spikes in B1 to 213 spikes in B2) and that similarly the response to AMPA was inhibited (from 401 spikes in B1 to 196 spikes in B2). The response to carbachol was unaffected.

Partial recovery from the effects of GSH (after 20 minutes) is shown in records A3 and B3.

FIGURE 16: *Histogram representing mean data: GSH inhibits EAA and 10ms air-jet responses in VB*

(overleaf)

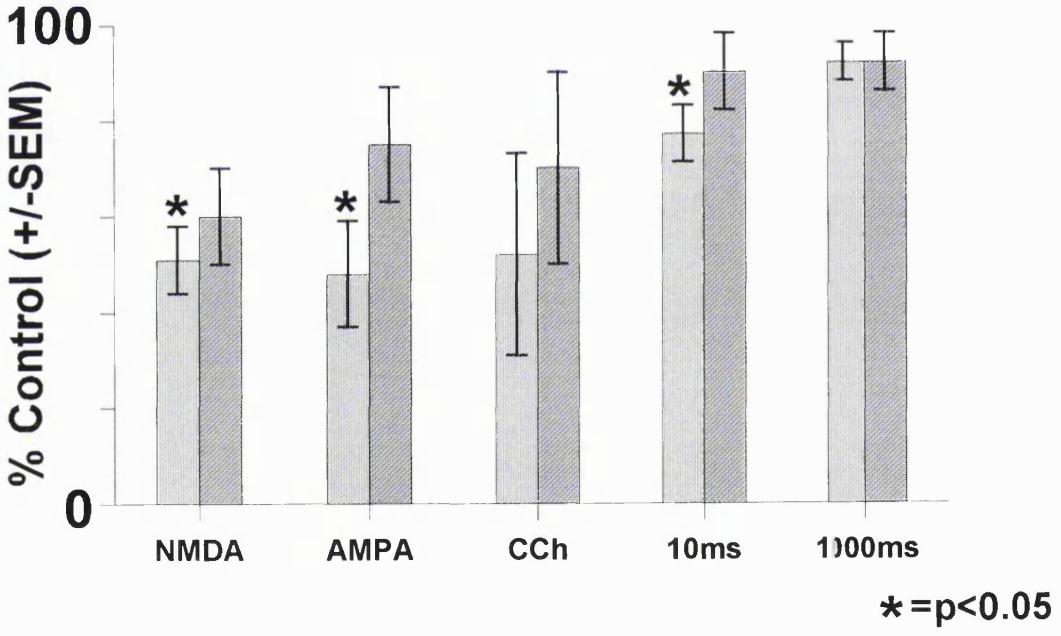

100
0
% Control (+/-SEM)
*
*
*
NMDA
AMPA
CCh
10ms
1000ms
*=p<0.05

FIGURE 16

Histogram representing mean data: GSH inhibits EAA and 10ms air-jet responses in VB

GSH was applied to 6 VB neurones; this histogram contains mean percentages of control responses for the effects of GSH on these 6 cells (light bars) and the effect of 20 minutes of recovery (dark bars). Error bars represent the standard error of the mean; asterisks indicate statistical significance, according to the Wilcoxon Signed Rank test ($p<0.05$).

The effects to be noted are GSH's inhibition of EAA responses and responses to 10ms air-jet vibrissal stimulation.

This data also appears in Figure 14 to enable comparison with the effects of GSNO.

TABLE 5

Summary of the effects of GSH on VB neurones (n=6)

	NMDA	AMPA	Cch	10ms	1000ms
GSH (-5 to 100nA)	51±7*	48±11*	52±21	77±6*	92±4

(mean % control ± standard error of mean)

* =$p<0.05$, Wilcoxon Signed Rank test

R.6.(iii). REDUCED GLUTATHIONE (GSH): dLGN NEURONES

GSH was applied to 7 dLGN neurones with iontophoretic currents in the range -5 to -50nA. These cells had not been used to test any of the other compounds under investigation.

In all cases, the response of the dLGN cells to NMDA was significantly ($p<0.05$, Wilcoxon Signed Rank test) inhibited during iontophoretic co-ejection of GSH, to $53 \pm 8\%$ of control (mean $\pm$ standard error of the mean) . Similarly, the response to AMPA was reduced, to $39 \pm 8\%$ of control ($p<0.05$).

The responses of 6 of the 7 cells to flash visual stimuli were reduced in the presence of GSH, to $70 \pm 7\%$ of control values ($p<0.05$). The visual responses of the remaining one cell were unaffected by the presence of GSH.

Figure 17 shows the effects of GSH on a typical dLGN neurone. Figure 18 shows the mean data for all 7 of the dLGN cells to which GSH was applied, which is also shown numerically in Table 6.

FIGURE 17: *GSH inhibits visual and EAA responses of a typical dLGN neurone*

(overleaf)

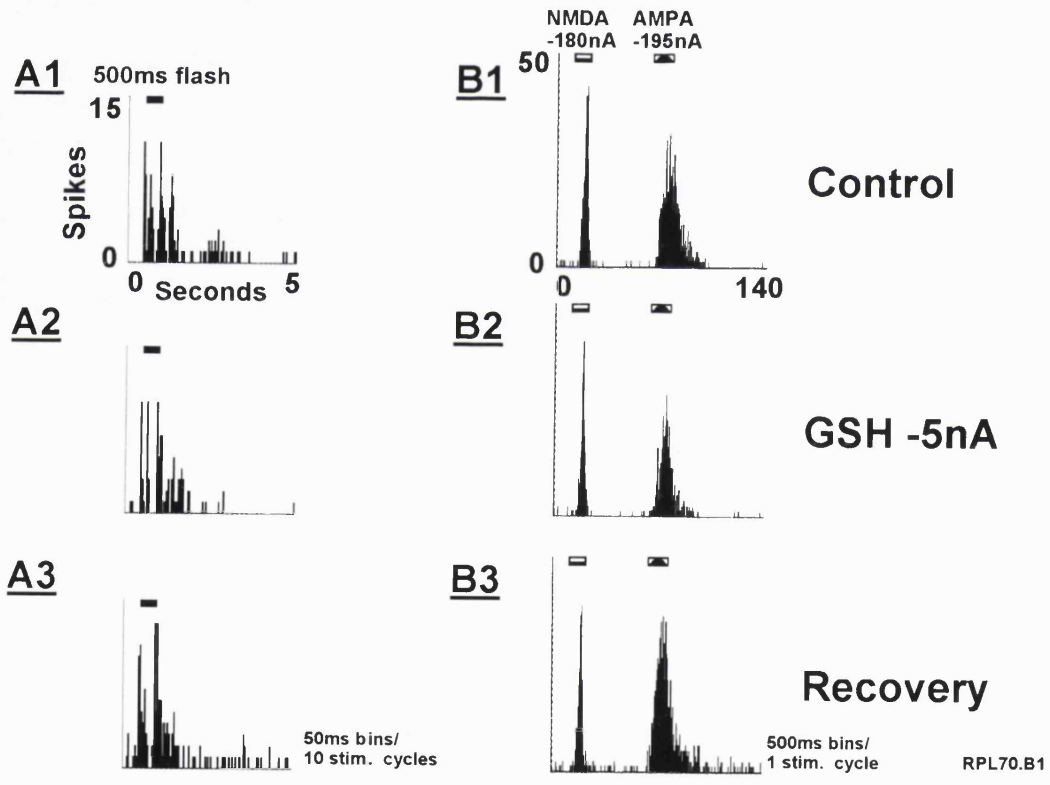

A1
500ms flash
15
Spikes
0
0
Seconds
5
A2
A3
50ms bins/
10 stim. cycles
B1
NMDA
-180nA
AMPA
-195nA
50
0
0
140
Control
B2
GSH -5nA
B3
Recovery
500ms bins/
1 stim. cycle
RPL70.B1

FIGURE 17

GSH inhibits visual and EAA responses of a typical dLGN neurone

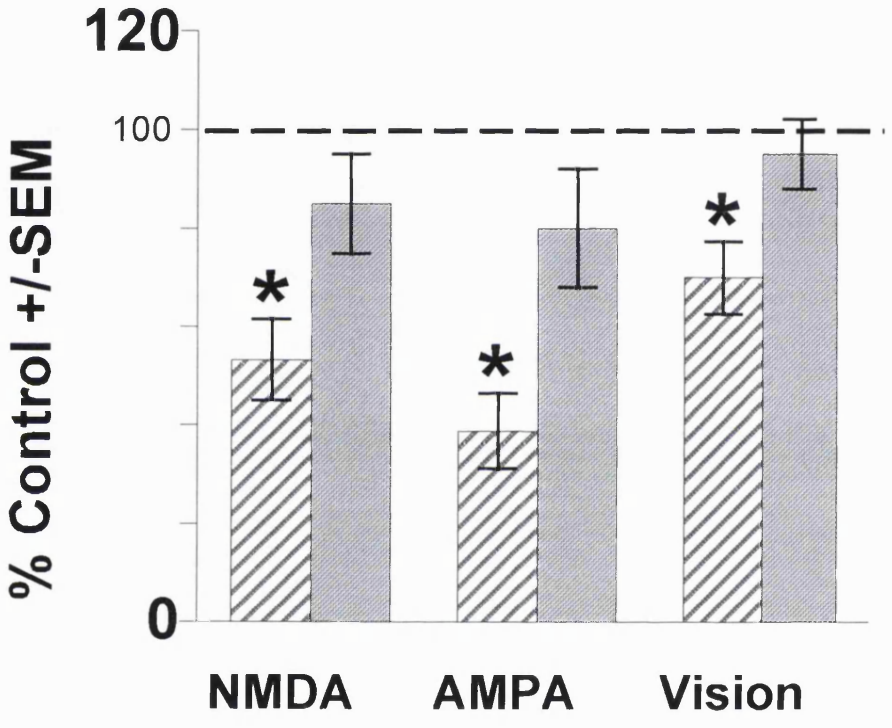
This figure shows PSTHs representing action potential firing of a single dLGN neurone, collected into bins and plotted against time.

Column A represents the cumulative responses of the cell to 10 presentations of a 500ms flash stimulus, in which the horizontal bar represents the duration of the stimulus. Record A2 shows the effects of GSH relative to the control response shown in A1; it can be seen that the spike response was reduced by GSH from 112 to 84 spikes. Recovery is shown in A3.

The right hand column, B, show the cell's responses to iontophoretically applied NMDA and AMPA; in this case, however, the records each represent a single cycle of stimulation. Note that the bin size differs from column A. It can be seen in B2 that the cell's response to NMDA was reduced relative to control (from 268 to 195 spikes) as was the response to AMPA (from 553 to 349 spikes). Recovery is shown in B3.

FIGURE 18: *Histogram representing mean data: GSH inhibits visual and EAA responses in dLGN*

(overleaf)



* =p<0.05

FIGURE 18

Histogram representing mean data: GSH inhibits visual and EAA responses in dLGN

This histogram shows the mean data for the 7 dLGN neurones to which GSH was applied. Light bars represent the mean percentage of control values following GSH application; dark bars represent the effects of 20 minutes of recovery. Error bars represent the standard error of the mean. Asterisks indicate statistical significance ($p<0.05$) according to the outcome of the Wilcoxon Signed Rank test.

Note that all responses shown here were reduced relative to the control level indicated by the dashed line.

TABLE 6

Summary of the effects of GSH on dLGN neurones (n=7)

	NMDA	AMPA	Vision
GSH	53±8*	39±8*	70±7*

-5 to -50nA
(mean % control ± standard error of mean)

* =p<0.05, Wilcoxon Signed Rank test

R.6.(iv). OXIDISED GLUTATHIONE (GSSG): VENTROBASAL THALAMIC NEURONES

GSSG was applied to 10 neurones in VB, distinct from those used for other experiments. The iontophoretic currents used were in the range -5 to -60nA.

The only statistically significant effect observed following GSSG application was a reduction in the magnitude of the response to NMDA, it being 70 $\pm$ 9% of its control value (data expressed as mean percent control $\pm$ SEM) ($p<0.05$). Responses to AMPA, carbachol and somatosensory stimulation exhibited no statistically significant changes when exposed to GSSG.

Figure 19 represents the agonist responses of a typical VB neurone to which GSSG was applied. Figure 20 contains the mean data for the 10 cells to which GSSG was applied in histogram form, while Table 7 contains the same mean data in a numerical format.

FIGURE 19: *GSSG inhibits the NMDA response of a typical VB neurone*

(overleaf)

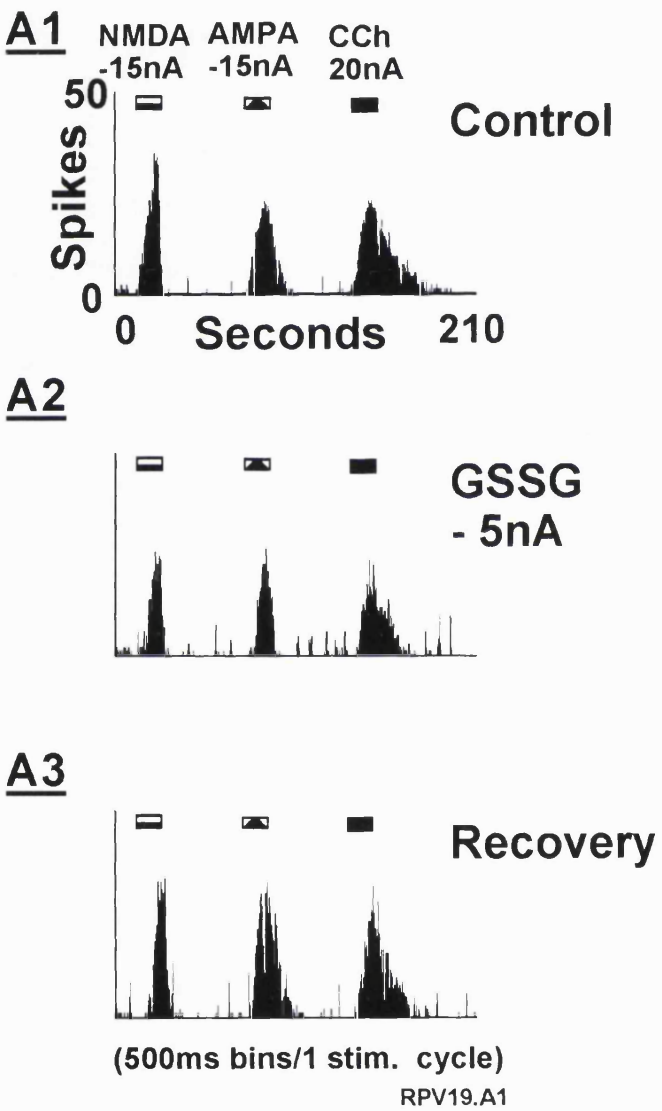

A1
NMDA
-15nA
AMPA
-15nA
CCh
20nA
50
Spikes
0
Control
0
Seconds
210
A2
GSSG
- 5nA
A3
Recovery
(500ms bins/1 stim. cycle)
RPV19.A1

FIGURE 19

GSSG inhibits the NMDA response of a typical VB neurone

This figure shows PSTHs representing spike firing of a single VB neurone in response to various iontophoretically applied agonists. Spikes are counted into bins and plotted against time. Symbols represent periods of agonist ejection.

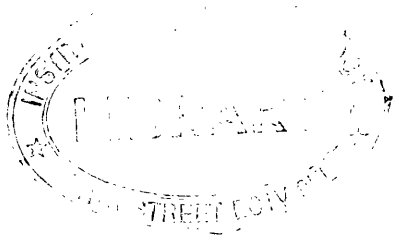
Record A1 represents control data; this can be compared with the effects of 20 minutes of GSSG ejection as shown in record A2. It can be seen that the response to NMDA is reduced in A2 (at 212 spikes) compared with that in A1 (510 spikes). The responses to AMPA and carbachol were relatively unaffected by GSSG application.

The effect of 28 minutes recovery from GSSG is shown in record A3.

Responses to somatosensory stimulation were unaffected by GSSG application and for this reason are not shown in this figure.

FIGURE 20: *Histogram representing mean data: GSSG inhibits NMDA responses in VB*

(overleaf)



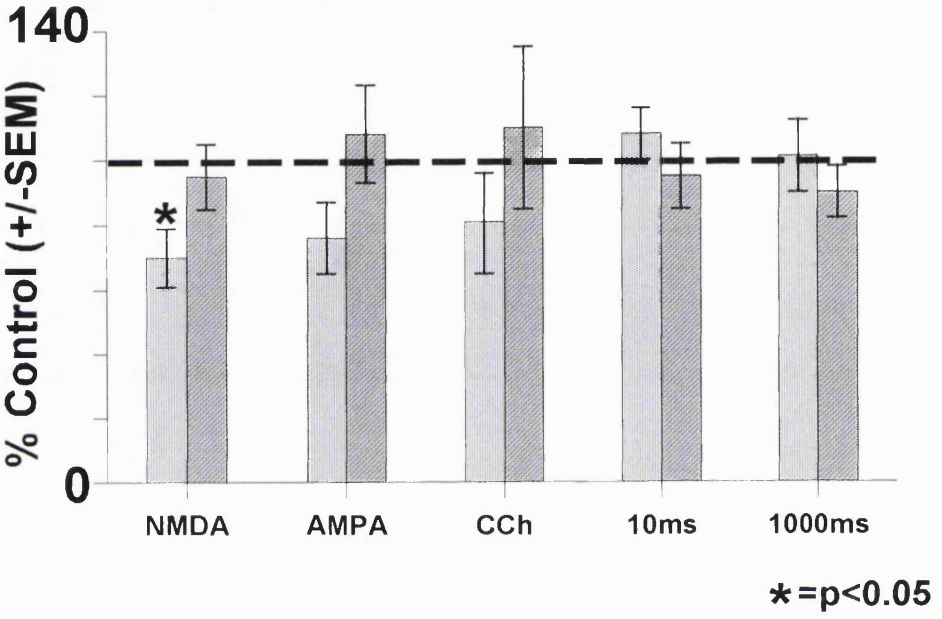

140
% Control (+/-SEM)
0
*
NMDA
AMPA
CCh
10ms
1000ms
*=p<0.05

FIGURE 20

Histogram representing mean data: GSSG inhibits NMDA responses in VB

GSSG was applied to 10 VB neurones; this histogram contains mean data for its effects on those 10 cells (light bars) and data after 20 minutes recovery (dark bars). Data are given as percentages of the control response; the error bars represent the standard error of the mean. The asterisk indicates statistical significance ($p<0.05$) according to the outcome of the Wilcoxon Signed Rank test.

The point to be noted from this data is the inhibition by GSSG of the cells' response to NMDA.

These data are also included in Figure 14 for comparison with the effects of GSNO.

TABLE 7

Summary of the effects of GSSG on VB neurones (n=10)

	NMDA	AMPA	Cch	10ms	1000ms
GSSG (-5 to 60nA)	70±9*	76±11	81±15	108±8	101±11

(mean % control ± standard error of mean)

* =$p<0.05$, Wilcoxon Signed Rank test

R.7. GLUTATHIONE: SUMMARY

Glutathione was applied to VB neurones in both its reduced and oxidised forms; the reduced form was also applied to dLGN neurones.

GSH, both in VB and in dLGN, produced an inhibition in the cells' responses to NMDA and AMPA, as well as in responses to vibrissal stimuli (VB) and visual stimuli (dLGN). This is not only evidence that GSNO is acting via nitric oxide production rather than via its glutathione moiety, but also is indicative of a possible role for glutathione as an inhibitory modulator of thalamic neurotransmission. There are a number of possible roles for glutathione described in the literature; these are discussed in more depth in section D.2.

Similar to GSH, GSSG was also found to inhibit NMDA responses in VB, but only a small effect on responses to AMPA was observed, which was not statistically signifcant. However, an effect solely on NMDA receptors has been described in the literature; this is discussed in section D.3.

R.8. L-ARGININE

R.8.(i). L-ARGININE: INTRODUCTION

L-arginine is an amino acid, and is the biosynthetic precursor of nitric oxide, as discussed in section I.4.(ii). and section I.4.(v).b. It was prepared as a 50mM aqueous solution at pH 4 and ejected as a positive ion.

R.8.(ii). L-ARGININE: dLGN NEURONES

L-arginine was applied to a total of 12 dLGN neurones with iontophoretic currents in the range of 5 to 20nA. None of these cells had previously been exposed to any of the other test compounds.

Responses to NMDA were significantly ($p<0.01$, Wilcoxon Signed Rank test) potentiated by L-arginine to $140 \pm 9\%$ of control values (expressed as mean percentage of the control response $\pm$ the standard error of the mean). Similarly, responses to AMPA were increased by L-arginine coapplication to $112 \pm 11\%$ of control ($p<0.05$). Visual responses were also potentiated during L-arginine ejection, to $146 \pm 9\%$ of control ($p<0.01$, $n=10$).

It was found that higher currents of L-arginine caused a secondary effect; in addition to the low current potentiation, higher currents of L-arginine were often found to inhibit the responses of the dLGN neurones. Figure 21 represents the responses of a typical dLGN neurone and shows both a potentiation of responses at a low iontophoretic current and an inhibition at a higher current. Figure 22 is a histogram representation of the mean potentiating effect of L-arginine on dLGN neurones, data which is also shown in Table 8.

FIGURE 21: *L-arginine potentiates visual and EAA responses of a dLGN neurone, but inhibits at a higher iontophoretic current*

(overleaf)

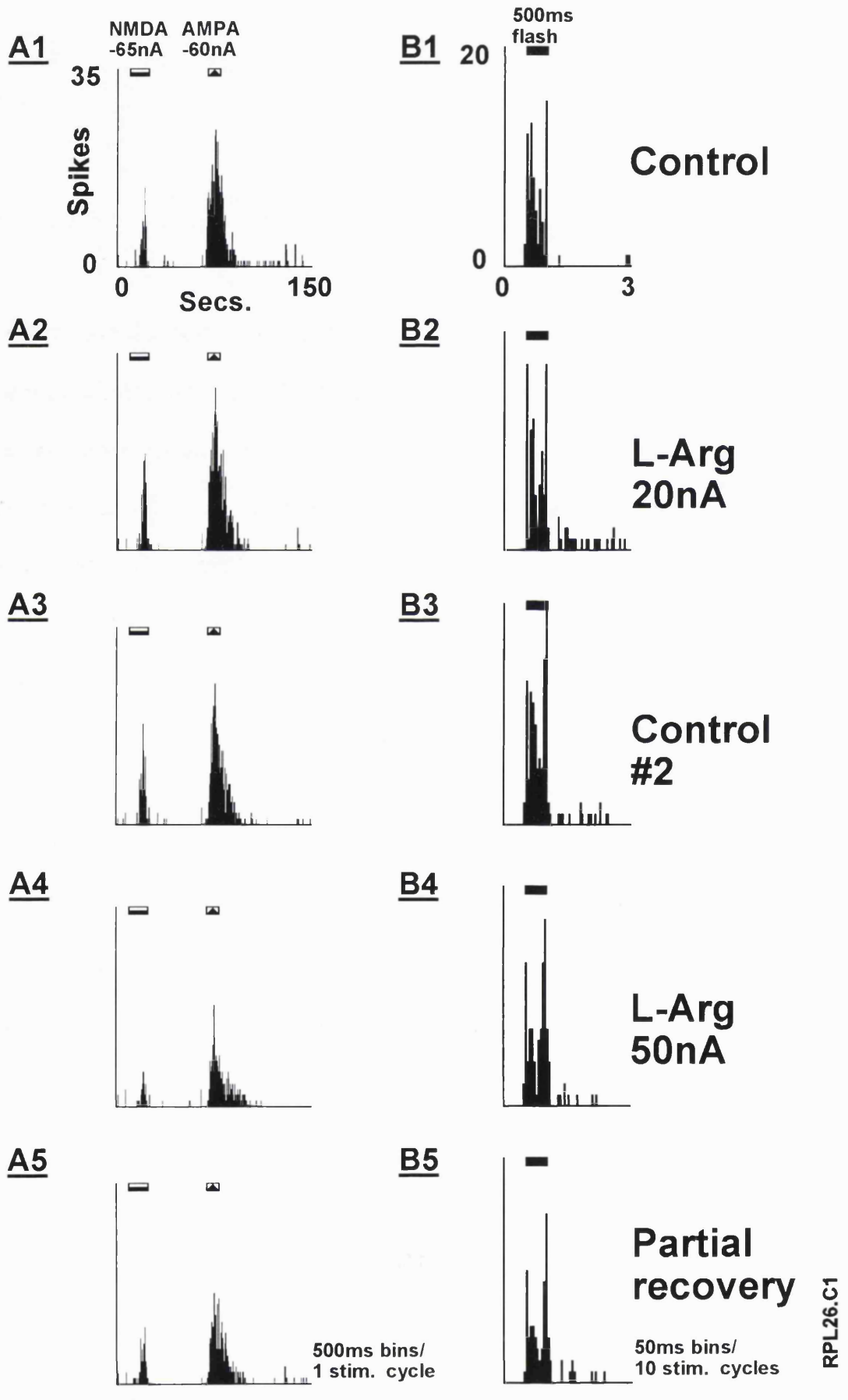

A1
NMDA -65nA
AMPA -60nA
35
Spikes
0
0
150
Secs.
B1
500ms flash
20
0
0
3
Control
A2
B2
L-Arg 20nA
A3
B3
Control #2
A4
B4
L-Arg 50nA
A5
B5
Partial recovery
500ms bins/ 1 stim. cycle
50ms bins/ 10 stim. cycles
RPL26.C1

FIGURE 21

L-arginine potentiates visual and EAA responses of a dLGN neurone, but inhibits at a higher iontophoretic current

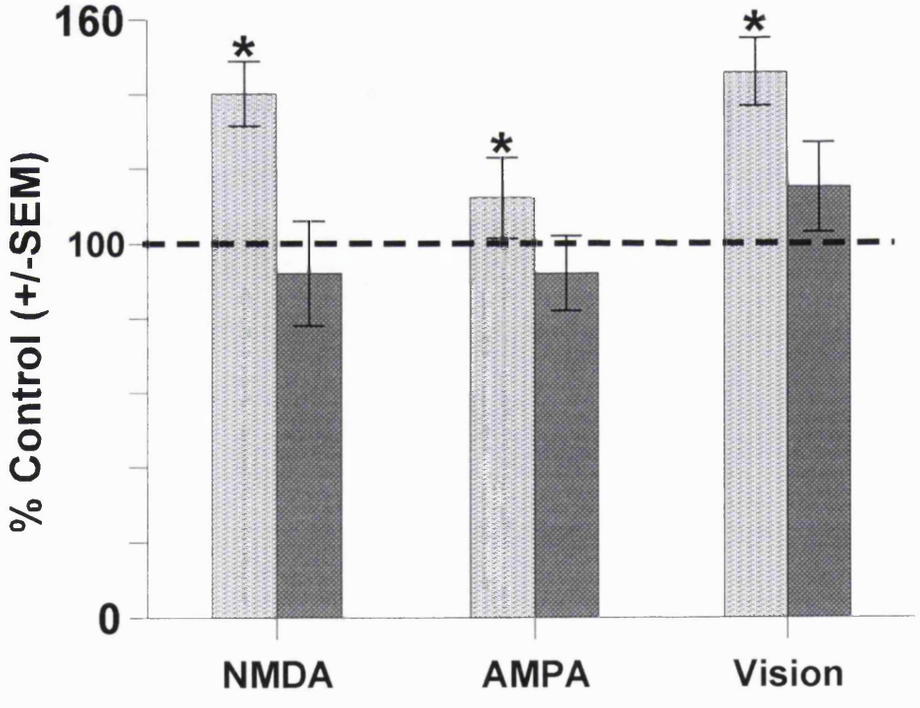
This figure shows PSTHs representing action potential spike responses of a single dLGN neurone collected into bins and plotted against time.

Column A shows records of the cell's responses to NMDA and AMPA. Each record represents a single cycle of stimulation; symbols represent periods of agonist ejection. Record A2 shows L-arginine at a relatively low iontophoretic current (20nA for 12 minutes) causing a potentiation of the response to NMDA (from 79 to 107 spikes) and of the response to AMPA (from 474 to 538 spikes). Following recovery, the same cell was exposed to 4 minutes of L-arginine at a higher iontophoretic current (50nA, record A4) causing an inhibition of responses to both NMDA and AMPA. Partial recovery from this effect is shown in A5, taken 4 minutes after the cessation of L-arginine ejection.

In a similar pattern, the effects of L-arginine on visual responses of the same dLGN neurone are shown in column B. In this column, the bin size is reduced for clarity and each record incorporates cumulative data from 10 stimulus presentations. In each case the horizontal bar represents the duration of the flash stimulus. Record B2 shows L-arginine's enhancement of the visual response after 4 minutes of ejection, to 97 spikes relative to the control of 74 spikes shown in B1. Similar to the pharmacological stimuli shown in column A, L-arginine caused an inhibition of the visual response when applied with a higher iontophoretic current, shown in record B4.

FIGURE 22: *Histogram representing mean data: L-arginine enhances visual and EAA responses in dLGN*

(overleaf)



* =p<0.05

FIGURE 22

Histogram representing mean data: L-arginine enhances visual and EAA responses in dLGN

This histogram contains mean data for the 12 dLGN cells to which L-arginine was applied (light bars, although n=10 for the visual responses) and the effect of 6 minutes of recovery (dark bars). Error bars represent the standard error of the mean. Asterisks indicate statistical significance ($p<0.05$), calculated by the Wilcoxon Signed Rank test.

L-arginine caused an enhancement of the cell's responses to all 3 stimuli.

TABLE 8

Summary of the effects of L-arginine on dLGN neurones

	NMDA	**AMPA**	**Vision**
L-arginine (5-12nA)	140±9* (n=12)	112±11* (n=12)	146±9* (n=10)

(mean % control ± standard error of mean)

* =$p<0.05$, Wilcoxon Signed Rank test

R.9. L-NITROARGININE METHYLESTER (L-NAME)

R.9.(i). L-NAME: INTRODUCTION

L-NAME is a derivative of L-arginine which can be used as a competitive inhibitor of nitric oxide synthase, as discussed in section I.4.(v).a. For the present experiments, it was prepared for iontophoresis in the same way as L-arginine, i.e. as a 50mM aqueous solution at pH4. It was ejected as a positive ion.

R.9.(ii). THE EFFECTS OF L-NAME ON dLGN NEURONES

L-NAME was applied to 7 dLGN neurones with iontophoretic currents in the range 20 to 100nA. None of these 7 cells had been used to test any other compound.

In 3 of the 7 neurones, L-NAME was found to inhibit responses to NMDA, but this effect was statistically non-significant, being a mean reduction to 88 $\pm$ 10% of control. Similarly, the cells' responses to AMPA were inhibited by L-NAME in 4 of the 7 neurones; this too was statistically non-significant (92 $\pm$ 11% of control). Visual responses were inhibited in 4 cases of the 7; as in the case of the pharmacological stimuli, there was no statistically significant change over the population from control values (116 $\pm$ 21%).

The mean data illustrating the lack of statistically significant effect of L-NAME is shown in histogram form in Figure 23 and numerically in Table 9.

FIGURE 23: *Histogram representing mean data: L-NAME has no effect on dLGN neurones*

(overleaf)

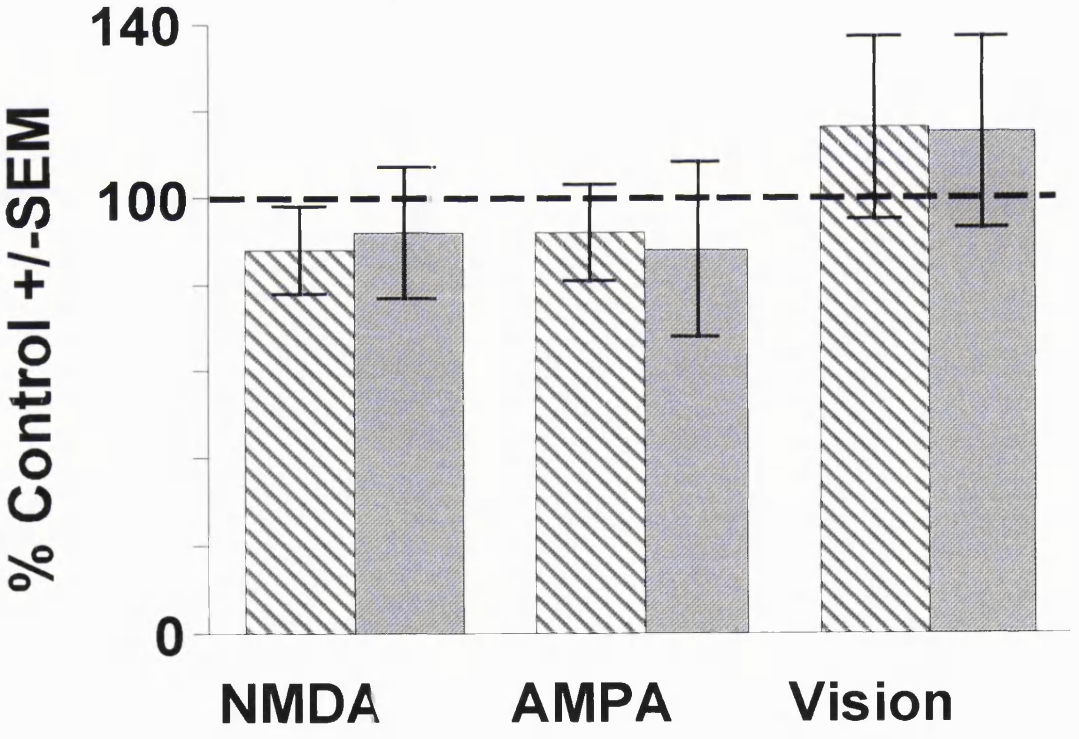

140
100
0
% Control +/-SEM
NMDA
AMPA
Vision

FIGURE 23

Histogram representing mean data: L-NAME has no effect on dLGN neurones

This histogram contains mean data for the 7 dLGN neurones to which L-NAME was applied with currents of 20 to 100nA. Light bars represent the mean percentage of control values following L-NAME ejection; dark bars indicate the effect of 6 minutes of recovery. Error bars represent the standard error of the mean.

No statistically significant effect was observed.

TABLE 9

Summary of the effects of L-NAME on dLGN neurones (n=7)

	NMDA	AMPA	Vision
L-NAME 20 to 100nA	88±10	92±11	116±21

(mean % control ± standard error of mean)

R.10. DRUGS AFFECTING NOS: SUMMARY

As predicted by the results of Do *et al* (1994) using a VB preparation, the precursor of nitric oxide, L-arginine, caused a potentiation of responses to NMDA, AMPA and visual stimulation. This result is probably due to the fact that the rate limiting step in the synthesis of nitric oxide is the availability of L-arginine, the substrate for nitric oxide synthase (Palmer *et al*, 1988b); thus increasing its availability increases the rate of production of nitric oxide. Thus the net effect is the same as follows the use of a nitric oxide donor, that is a potentiation of sensory and EAA responses.

Conversely, if the availability of nitric oxide is reduced by inhibition of nitric oxide synthesis using L-NAME, it might be expected that an inhibition of sensory and EAA responses would follow. However, this was not the case in the present experiments; L-NAME failed to produce any effect. The explanation for the lack of effect of L-NAME is probably that the extent of tonic nitric oxide release was low or that the conditions affecting the relay cell membranes were such that nitric oxide was having little influence. These possibilities are more thoroughly discussed in section D.2.(i). It is also possible that a higher iontophoretic current of L-NAME (greater than the 100nA used in these experiments) may produce an effect.

Despite L-NAME's failure to elicit an effect under the protocol used for the experiments detailed above, it can be hypothesised that similarly to the results of Do *et al* (1994) in VB, L-NAME would act to prevent the potentiation of responses in dLGN which followed L-arginine application if the two compounds were applied simultaneously.

R.11. 8-BROMO CYCLIC GMP

R.11.(i). 8-Br cGMP: INTRODUCTION

8-Br-cGMP is an analogue of the intracellular cyclic nucleotide second messenger cGMP (see section I.4.(v).e). It is membrane permeant, and as such can be applied extracellularly by iontophoresis. The methodology used was adapted from that of Hentall (1995), although a much lower concentration (50mM in water at pH 8) was found to be effective.

R.11.(ii). 8-Br-cGMP AND VENTROBASAL THALAMIC NEURONES

8-Br-cGMP was applied to 10 ventrobasal thalamus neurones, none of which had previously been exposed to any of the other compounds under investigation. Iontophoretic currents in the range -30 to -80nA were used, with a retaining current of 15nA. Effects of the drug were observed following 8 - 20 minutes of iontophoretic ejection at these current amplitudes.

The mean response to NMDA ($\pm$ the standard error of the mean) was increased by coapplication of 8-Br-cGMP to 138 $\pm$ 14% ($p<0.05$, Wilcoxon Signed Rank test), while that to AMPA was similarly elevated to 149 $\pm$ 13% of control values ($p<0.05$). The mean response to 10ms air-jet vibrissal stimulation was increased by 8-Br-cGMP ejection to 274 $\pm$ 76% of control ($p<0.05$, $n=8$) while the mean response to a longer duration (1000ms) air-jet pulse was relatively unaffected, being increased to only 107 $\pm$ 10% of control (non-significant).

The peristimulus time histograms shown in Figure 24 represent the responses of a

typical ventrobasal thalamus cell to which 8-Br-cGMP was applied; Figure 25 shows mean data from the population of 10 VB neurones to which the drug was applied. The mean data are also shown in tabulated form in Table 10.

FIGURE 24: *8-Br-cGMP potentiates the responses of a VB cell to EAA agonists and air-jet stimulation*

(overleaf)

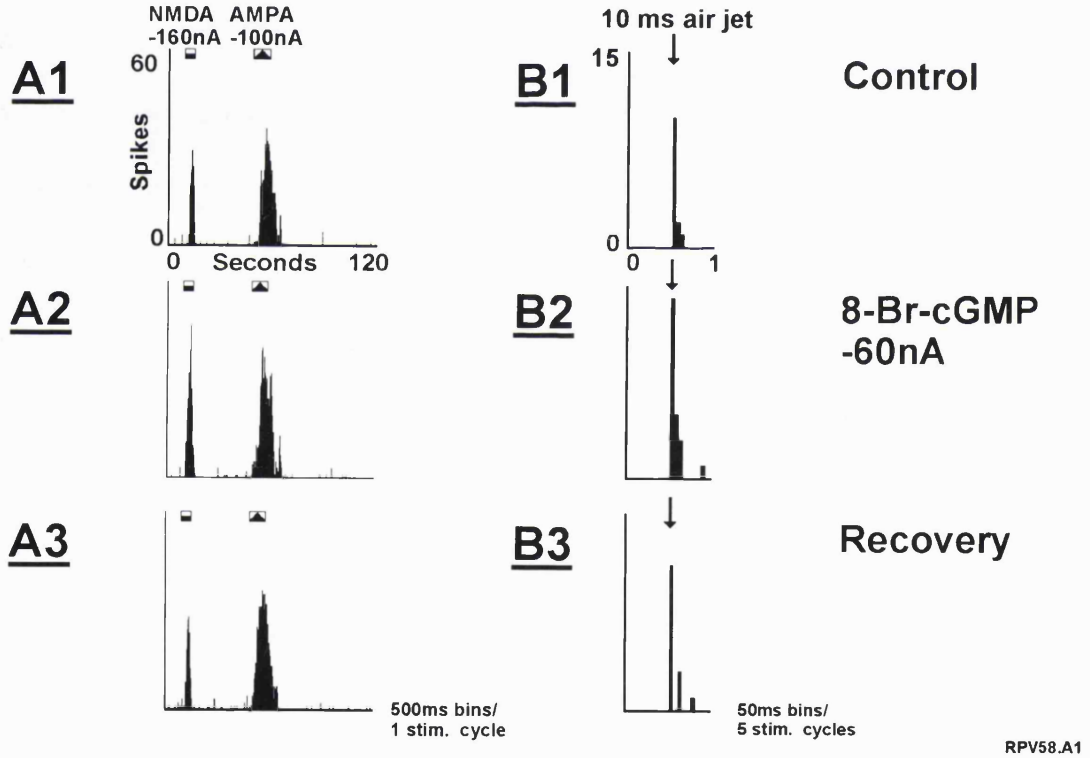

NMDA AMPA
-160nA -100nA
A1
60
Spikes
0
0 Seconds 120
A2
A3
500ms bins/
1 stim. cycle
10 ms air jet
B1
15
0
0 1
Control
B2
8-Br-cGMP
-60nA
B3
Recovery
50ms bins/
5 stim. cycles
RPV58.A1

FIGURE 24

8-Br-cGMP potentiates the responses of a VB cell to EAA agonists and air-jet stimulation

The figure shows PSTHs representing action potential spike counts of a single VB neurones collected in to bins and plotted against time.

Column A shows response of the cell to stimulation by the excitatory amino acid agonists NMDA and AMPA; symbols represent periods of agonist ejection. Control responses are shown in record A1, while responses following 20 minutes of 8-Br-cGMP ejection are shown in record A2. It can be seen that the response to NMDA was increased (from 192 to 352 spikes) as was that to AMPA (624 to 869 spikes). Recovery from the effects of 8-Br-cGMP is shown in record A3, 20 minutes after the cessation of ejection.

Column B similarly shows the effects of 20 minutes of 8-Br-cGMP application on the responses of the same VB neurone to 5 cycles of air-jet vibrissal stimulation, cumulated and on an expanded timebase. In each record the arrow indicates the onset of the stimulus. It can bee seen in record B2 that the response to the air-jet is increased relative to the control record shown in B1 (from 13 to 23 spikes). Recovery from the effects of 8-Br-cGMP is shown in B3.

FIGURE 25: *Histogram representing mean data: 8-Br-cGMP enhances sensory and EAA responses in VB*

(overleaf)

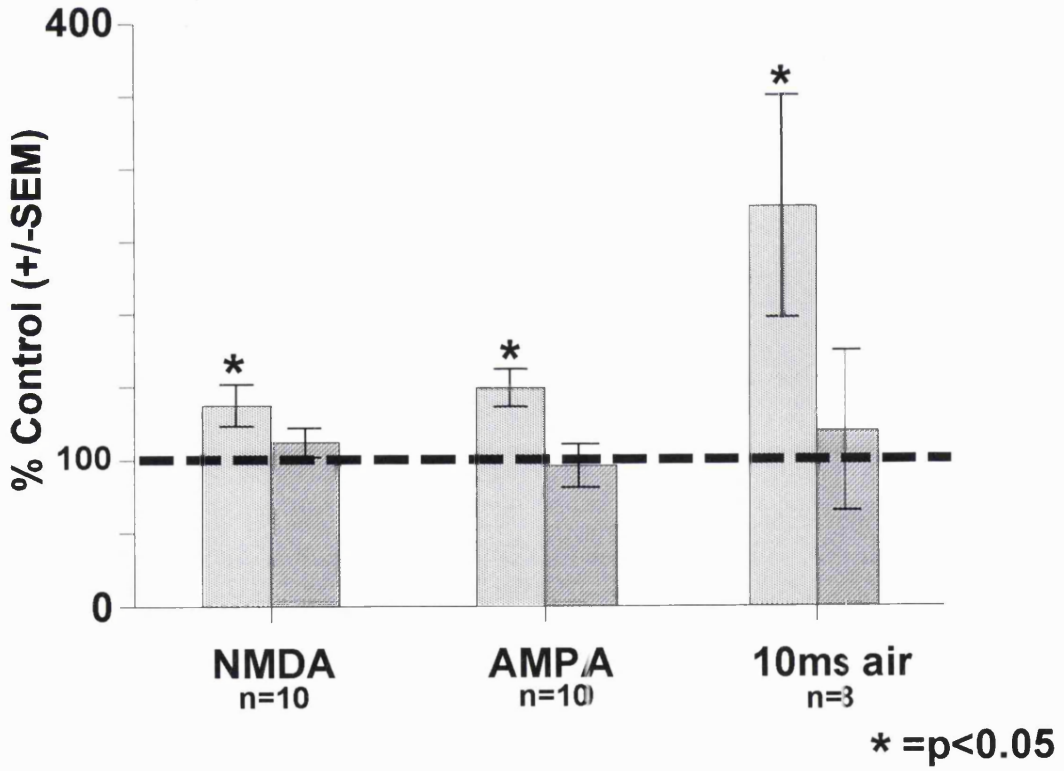

400
100
0
% Control (+/-SEM)
*
*
*
NMDA
n=10
AMPA
n=10
10ms air
n=3
* =p<0.05

FIGURE 25

Histogram representing mean data: 8-Br-cGMP enhances sensory and EAA responses in VB

8-Br-cGMP was applied to 10 VB neurones, and the mean effects are represented in this histogram as percentages of control values (light bars); dark bars indicate the effects of 12 minutes of recovery. Error bars represent the standard error of the mean. Asterisks indicate statistical significance ($p<0.05$, Wilcoxon Signed Rank test).

It can be seen that the responses of the VB neurones to both NMDA and AMPA were significantly increased relative to control values, as were responses to 10ms air-jet vibrissal stimulation (n=8 in this case).

TABLE 10

Summary of the effects of 8-Br-cGMP on VB neurones

	NMDA	AMPA	10ms	1000ms
8-Br-cGMP	138±14* (n=10)	149±13* (n=10)	274±76* (n=8)	107±10 (n=10)

(mean % control ± standard error of mean)

* =$p<0.05$, Wilcoxon Signed Rank test

R.11.(iii). 8-Br-cGMP: dLGN NEURONES

8-Br-cGMP was applied to 8 dLGN neurones to which none of the other test compounds had been applied. Iontophoretic currents of between -40 and -75nA were used with a retaining current of 15nA. As with VB cells, effects of 8-Br-cGMP were observed 8 - 20 minutes following the onset of ejection.

The response to NMDA was potentiated by 8-Br-cGMP to 254 $\pm$ 59% of control values (mean $\pm$ standard error of the mean) while the mean response to AMPA was similarly increased to 225 $\pm$ 45%. Both were statistically significant ($p<0.05$) increases (Wilcoxon Signed Rank test). The mean response to visual stimulation was increased by 8-Br-cGMP ejection to 217 $\pm$ 69% of control values ($p<0.05$).

A representative dLGN cell to which 8-Br-cGMP was applied is shown in Figure 26. Mean data for the application of 8-Br-cGMP to dLGN cells is shown in histogram form in Figure 27 and in numeric form in Table 11.

FIGURE 26: *8-Br-cGMP potentiates the responses of a dLGN cell to EAA agonists and visual stimulation*

(overleaf)

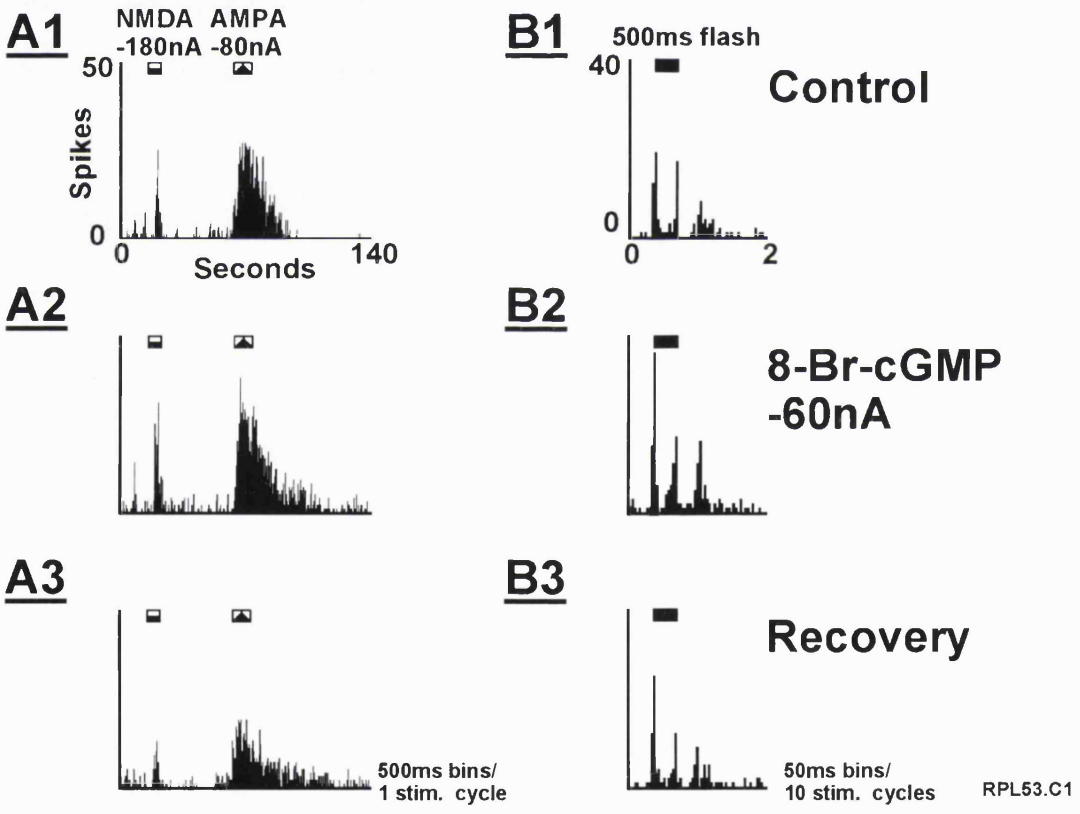

A1
NMDA AMPA
-180nA -80nA
50
Spikes
0
0
Seconds
140
A2
A3
500ms bins/
1 stim. cycle
B1
500ms flash
40
0
0
2
Control
B2
8-Br-cGMP
-60nA
B3
Recovery
50ms bins/
10 stim. cycles
RPL53.C1

FIGURE 26

8-Br-cGMP potentiates the responses of a dLGN cell to EAA agonists and visual stimulation

This figure shows the effects of 8-Br-cGMP on the action potential spike counts of a single neurone in dLGN. Spikes are shown grouped into bins and plotted against time.

Column A shows the effects of 8-Br-cGMP on responses of the cell to NMDA and AMPA; symbols represent periods of agonist ejection. Record A1 contains control data, record A2 data in the presence of 8-Br-cGMP (following 12 minutes of its ejection) and record A3 data following 20 minutes of recovery. It can be seen that 8-Br-cGMP caused an enhancement of responses both to NMDA (102 to 204 spikes) and to AMPA (891 to 1208 spikes).

In a similar manner, column B shows the effect of 8-Br-cGMP on visual responses of the same dLGN neurone. Bars represent the duration of the flash stimulus; for these records, 10 presentations of the stimulus were cumulated and the data displayed on an timebase expanded relative to that used for column A. In record B2 it can be seen that the response to the flash stimulus was increased by 20 minutes of iontophoretic application of 8-Br-cGMP relative to the control response, i.e. from 89 to 152 spikes. The response of the neurone to the flash stimulus following 8 minutes of recovery from the effects of 8-Br-cGMP is shown in record B3.

FIGURE 27: *Histogram representing mean data: 8-Br-cGMP enhances visual and EAA responses in dLGN*

(overleaf)

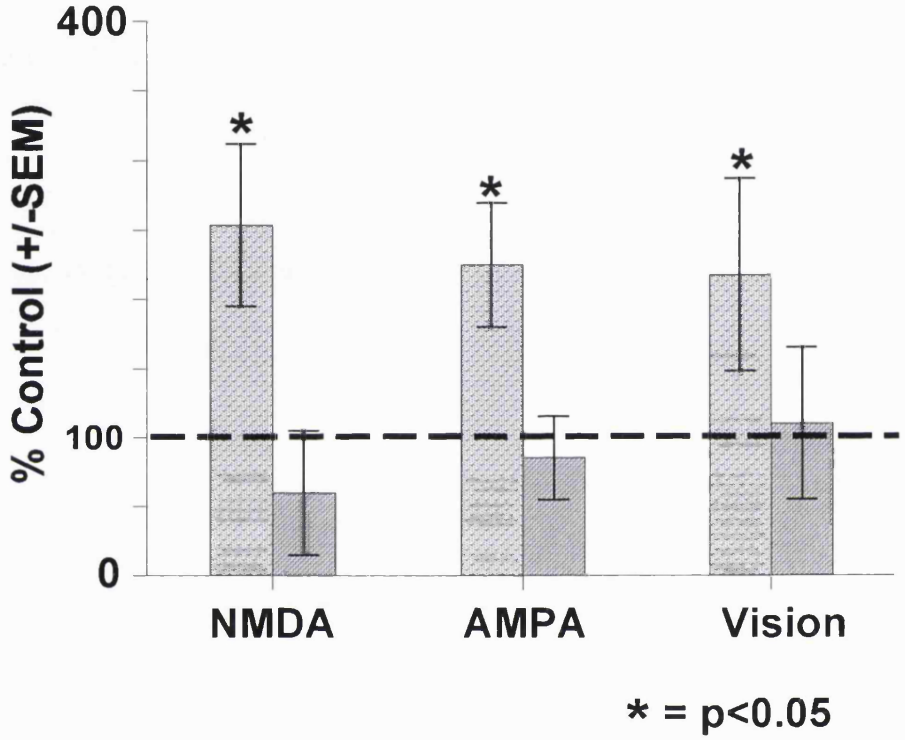

400
100
0
% Control (+/-SEM)
*
*
*
NMDA
AMPA
Vision
* = p<0.05

FIGURE 27

Histogram representing mean data: 8-Br-cGMP enhances visual and EAA responses in dLGN

This histogram summarises the effects of 8-Br-cGMP on 8 dLGN neurones; light bars represent percentages of control values while error bars represent plus or minus the standard error of the mean. Dark bars show the effects of 8 minutes of recovery. Asterisks indicate statistical significance ($p<0.05$, Wilcoxon Signed Rank test).

The histogram reveals that responses to NMDA, AMPA and visual stimulation were all significantly raised above control levels in the presence of 8-Br-cGMP.

TABLE 11

Summary of the effects of 8-Br-cGMP on dLGN neurones (n=8)

	NMDA	**AMPA**	**Vision**
8-Br-cGMP	254±59*	225±45*	217±69*

(mean % control ± standard error of mean)

* =p<0.05, Wilcoxon Signed Rank test

R.12. 1-H-[1,2,4]-oxadiazolo-[4,3,a]-quinoxalin-1-one (ODQ)

ODQ is an inhibitor of soluble guanylate cyclase, one of the possible sites of action of nitric oxide. It has been used with some success in *in vitro* preparations, but has not to date been used in iontophoretic experiments.

A number of attempts to prepare solutions of ODQ for iontophoresis were made. ODQ is essentially insoluble in water or saline, and does not, therefore, fit the criteria usually applied to compounds to test their suitability for iontophoretic experiments. Despite this, however, it was thought that if by some means ODQ could be iontophoresed, it would prove invaluable in the elucidation of the downstream effects of nitric oxide in the thalamus. If ODQ was used in combination with a nitric oxide donor and 8-bromo-cGMP, ODQ would be able to provide information regarding the mechanism of action of the donor; if the nitric oxide donor was acting to stimulate soluble guanylate cyclase to increase the synthesis of cGMP, then its effects should be blocked by coapplication of ODQ, while the effects of 8-bromo-cGMP should not.

ODQ is soluble in dimethylsulphoxide (DMSO) and in ethanol. Initial experiments attempted to form a 50mM solution of ODQ in absolute ethanol, which could then be diluted with water to finally obtain a 10mM ODQ solution. This proved to be unsuccessful, however, as a precipitate was formed when water was added to the ODQ/ethanol solution. Attempts to iontophorese ODQ at 10mM in absolute ethanol were also unsuccessful, due to excessive barrel resistance.

Similar attempts were made to make solutions of ODQ in DMSO or a combination of DMSO and water; these were also unsuccessful as attempting to iontophorese the solution resulted in damage to the cell, such that, in some cases, erratic firing was observed.

In all cases, the end result was loss of responses of the cell to all stimuli (Figure 28).

These unfortunate but not entirely unexpected failures led to the decision to abandon attempting to apply ODQ by this method; while undoubtedly the results would have been interesting and important, it seems that ODQ is a compound which is not suitable for iontophoretic experiments.

FIGURE 28: *ODQ in DMSO causes loss of respsonsiveness of a VB neurone to air-jet stimuli*

(overleaf)

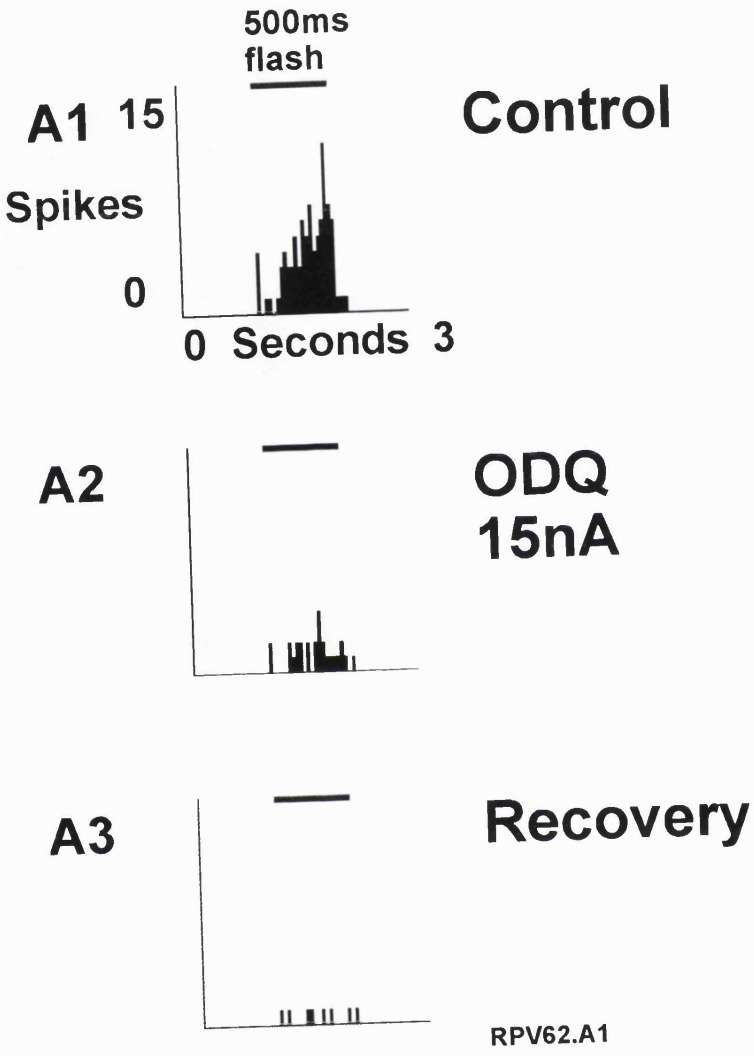

500ms
flash
A1
15
Spikes
0
0 Seconds 3
Control
A2
ODQ
15nA
A3
Recovery
RPV62.A1

FIGURE 28

ODQ in DMSO causes loss of responsiveness of a VB neurone to air-jet stimuli

This figure illustrates the effect of an attempt to iontophorese ODQ, dissolved in DMSO, onto a single VB neurone. Each record is a PSTH representing cumulative action potential firing of the cell over five cycles of stimulation. The horizontal bars represent the duration of the air-jet stimuli. Record A1 represents control data; A2 represents data recorded during ODQ ejection; A3 represents data recorded 20 minutes after ejection ceased. The result was a rapid and irreversible loss of all responses following ODQ ejection.

R.13. DRUGS AFFECTING cGMP: SUMMARY

One of the principal modes of action of nitric oxide is the stimulation of soluble guanylate cyclase to increase the rate of production of cGMP. As such, either inhibiting this enzyme or mimicking the effects of its product will provide important information regarding the effects of nitric oxide.

It was unfortunate, therefore, that it was not possible to apply ODQ by iontophoresis. As an inhibitor of soluble guanylate cyclase, ODQ may have provided information regarding the effects of removing one of nitric oxide's means of exerting its effects. It may have been the case however, that ODQ would have been subject to the same problems encountered with inhibitors of nitric oxide synthase, namely a lack of effect possibly due to an inactive nitric oxide system or a reduced salience of that system.

By contrast, 8-Br-cGMP acts to mimic the effects of the product of soluble guanylate cyclase, cGMP. Modifications of the cGMP molecule confer 8-Br-cGMP with membrane permeability, therefore allowing it to be applied extracellularly by iontophoresis but to have an intracellular site of action. The effects of 8-Br-cGMP in the present experiments were similar to those of nitric oxide donor compounds, i.e. an enhancement of sensory and EAA responses, both in VB and in dLGN. The means by which cGMP or 8-Br-cGMP may cause these effects are discussed in section D.2.(iii).

CHAPTER 4

DISCUSSION

D.1. GENERAL DISCUSSION

This thesis has demonstrated novel findings concerning the role of nitric oxide in thalamic sensory relay nuclei, and, additionally, has detailed for the first time the effects of glutathione on relay neurones in the same thalamic nuclei. In both cases the chosen experimental protocol was drug application by microiontophoresis to single ventrobasal thalamus or lateral geniculate nucleus neurones in an *in vivo* rat preparation. The use of iontophoretic drug application enabled drug application to be restricted to a localised area while recording the effects of the drug on a single neurone.

D.2. NITRIC OXIDE

The role of nitric oxide in thalamic sensory relay nuclei has received attention principally from three groups of researchers, all of whom have drawn a broadly similar conclusion from differing experimental approaches. The first description of a putative role for nitric oxide in the thalamus came from the work of Pape and Mager (1992), who used intracellular recording *in vitro* to identify the ability of nitric oxide donor compounds to influence the postsynaptic membrane so as to cause a depolarisation; this was thought to be a mechanism mediated intracellularly by cyclic GMP. Cudeiro *et al* (1994a&b) used a feline *in vivo* dLGN preparation and extracellular recording to show an inhibition of responses caused by blockade of nitric oxide synthesis, while Do *et al* (1994) boosted nitric oxide synthesis by increasing the availability of its precursor, L-arginine. These studies will be discussed in greater depth in subsequent sections to

compare their results with those of the present study, but in each case there appeared to be an excitatory action of nitric oxide on thalamocortical relay cells, whether rodent or feline and whether visual or somatosensory. As a consequence, when the present study was undertaken, it was hypothesised that manipulation of the nitric oxide system by a variety of pharmacological means should also reveal an excitatory action.

As detailed in Section I.4.(v)., there are numerous ways in which the biosynthesis, availability and downstream effects of nitric oxide can be pharmacologically manipulated. Some of these experimental techniques are applicable to the use of iontophoretic drug application, and will be discussed individually below.

D.2.(i). *Drugs Influencing Nitric Oxide Synthase: L-Arginine and L-NAME*

The synthesis of nitric oxide can be influenced in two ways. The first method involves boosting the concentration of nitric oxide's precursor, L-arginine, the availability of which is known to be rate limiting in the function of nitric oxide synthase (Palmer *et al*, 1988b). Alternatively, nitric oxide synthase can be competitively inhibited by a variety of compounds. Clearly, both of these experimental approaches are suitable for iontophoretic experiments, and both techniques were used to some extent in the present study.

Do *et al* (1994) used iontophoretic application of L-arginine to single VB neurones, and demonstrated that the neurones' responses to air-jet vibrissal stimulation were potentiated during L-arginine ejection, as were responses to the iontophoretically applied excitatory amino acid agonists NMDA and AMPA. The authors concluded that what was being observed was indeed an increase in the rate of operation of nitric oxide synthase in response to increased availability of its substrate. The present study therefore

aimed to investigate whether similar effects of L-arginine could be observed following its application to dLGN neurones.

The results showed that L-arginine does have a potentiating effect when applied to dLGN neurones with low iontophoretic currents; as discussed by Do *et al* (1994) with reference to VB neurones, however, L-arginine had a biphasic effect on dLGN relay neurones. This biphasic effect operated such that while low iontophoretic currents produced a short-lived potentiation of responses to visual stimuli and iontophoretic NMDA and AMPA stimulation, higher iontophoretic currents had the opposite, i.e. an inhibitory effect, causing reductions in the magnitudes of the cells' responses. In some cases, the secondary, inhibitory effect could also be observed following prolonged L-arginine ejection; in such circumstances, a potentiation occurred shortly after the onset of L-arginine ejection with an inhibition following some minutes later. Thus it can be concluded that L-arginine has two distinct effects on thalamic neurones, in a concentration dependent manner. Given the similarity of the low current potentiating effect to the effects of nitric oxide donors also described in this thesis, and to the effects described by Do *et al* (1994) in which the L-arginine-induced potentiations could be prevented by co-application of a nitric oxide synthase inhibitor, the results imply that this phenomenon is caused by an increase in the rate of synthesis of nitric oxide.

The mechanism of the secondary, inhibitory effect of iontophoretically applied L-arginine observed in this study and that of Do *et al* (1994) is less clear, but seems to be unrelated to nitric oxide production, as iontophoretically applying nitric oxide donor compounds at high currents (in excess of 200nA) failed to produce any effects additional to those observed at lower currents. A recent finding by Shen *et al* (1997) in dopaminergic neurones has suggested one possible explanation for L-arginine's inhibitory

effect, that being an inhibition of the function of a GABA transporter by L-arginine, independent of nitric oxide production. In this way, L-arginine potentiates the effects of GABA-mediated inhibition. A further possibility is incorporation of L-arginine into kyotorphin (L-tyrosyl-L-arginine), a dipeptide present in the CNS which can release Met-enkephalin; this system has been described in the spinal cord (Kawabata *et al*, 1993) in which L-arginine exerts both pro-nociceptive and anti-nociceptive actions, via the guanylate cyclase/cGMP pathway and the kyotorphin/Met-enkephalin pathway, respectively. As such, it is conceivable that L-arginine could have a similar dual role in other systems.

Conversely, the rate of synthesis of nitric oxide can be pharmacologically reduced using nitric oxide synthase inhibitors. This methodology has frequently been used in the investigation of nitric oxide systems including in the thalamus (Cudeiro *et al*, 1994a&b; Cudeiro *et al*, 1996). The studies of Cudeiro and coworkers utilised the L-arginine analogues N^G-monomethyl L-arginine and N^G-nitro-L-arginine as competitive NOS inhibitors and found consistently that iontophoretic application to feline dLGN relay neurones resulted in a reduction in the cells' responses to visual stimuli. However, Do *et al* (1994) observed a dissimilar effect of another arginine-analogue NOS inhibitor, L-NAME, in their rat VB preparation. Do and coworkers demonstrated that L-NAME by itself had very little effect on VB relay neurones, causing only a small inhibition in somatosensory and EAA responses, but that L-NAME when coapplied with L-arginine could prevent the latter's potentiating effects.

Reminiscent of the findings of Do *et al* (1994), the present study failed to demonstrate any consistent effect of L-NAME on rat dLGN relay neurones. There are several possible explanations for the differing effects of Cudeiro *et al* (1994a), Do *et al*

(1994) and the present study. Firstly there is the obvious possibility of a species difference: Cudeiro's study used cats, while Do and coworkers used rats. Secondly, there is a strong possibility that the basal level of nitric oxide synthesis is not consistent. The implication of the work of Cudeiro *et al* (1994a) is that the iontophoretically applied NOS inhibitors were reducing or eliminating the contribution of endogenous nitric oxide to the function of the dLGN relay cells. If, however, under some circumstances the contribution of endogenous nitric oxide is reduced or it becomes less important, then the use of NOS inhibitors would fail to resolve any effect. It is conceivable that, for instance, the level of anaesthesia or the chosen anaesthetic regimen could influence the activity of neurones in the brainstem (which are known to be involved in the control of attentiveness, Steriade *et al*, 1990) and as a consequence influence the activity of NOS located in the thalamic terminations of those brainstem neurones (Bickford *et al*, 1993). This is a testable hypothesis in that the chosen anaesthetic could be changed (for instance from urethane to halothane in the rat preparation) or the depth of anaesthesia could be varied. The use of NOS inhibitors for the investigation of the role of nitric oxide in the thalamus has been, therefore, only sporadically successful. Although this experimental technique has the advantage of influencing only the endogenous synthesis of nitric oxide and is therefore of great physiological relevance, it has the disadvantage that nitric oxide may be of importance under some circumstances which may be missed if the wrong experimental protocol is chosen, as may have been the case in the present study, in which it would have been more useful to test the effects of NOS inhibitors during a potentiation induced by L-arginine, similar to the experiments carried out by Do *et al* (1994). Furthermore, it remains an uninvestigated possibility that the firing state of the thalamic relay cells could influence the effectiveness of any endogenously released nitric oxide

which may be present; if the cell membrane is relatively hyperpolarised (such as occurs during burst firing mode), nitric oxide could be relatively less effective at enhancing responsiveness than at more positive membrane potentials. Under these circumstances, the use of NOS inhibitors may fail to reveal any effect.

Some alternative NOS inhibitors have recently become available; 7-nitroindazole (7-NI and its sodium salt 7-NINA) and TRIM have been used with some success, although not as yet in the thalamus. As discussed in section I.4.(v).a, 7-NI and TRIM are selective for the neuronal isoform of NOS (Moore *et al*, 1993; Handy and Moore, 1997), but this property would probably not rectify the problem described above, which does not arise because of a lack of isoenzyme selectivity. Additionally, 7-NI or 7-NINA may in fact have selectivity for the neuronal isoform of NOS conferred upon them merely by a difference in the accessibility of this form versus the endothelial form (Moore *et al*, 1993).

D.2.(ii). *Nitric Oxide Donor Compounds*

As a more direct experimental means of elevating the availability of nitric oxide to thalamic relay neurones, it is possible (as described in section I.4.(v).c.) to use nitric oxide donor compounds to deliver molecules of nitric oxide to their site of action. Such compounds take the form of carrier molecules, which may or may not be biologically active, which have nitric oxide molecules as part of their chemical structure or as an adjunct to it; they decompose to release nitric oxide either spontaneously or in reaction to exposure to biological media.

For the present study, three different nitric oxide donor compounds were used. It was thought that to utilise more than a single nitric oxide donor would help the

interpretation of the results; following the breakdown of such compounds, there may be effects mediated by the non-nitric oxide part of the molecule, and so experimentally to produce similar results from compounds with dissimilar chemical structures would implicate nitric oxide as the cause of any observed effects. The chosen compounds were sodium nitroprusside (SNP), 3-morpholinosydnonimine (SIN-1) and S-nitrosoglutathione (GSNO); each will be considered individually in subsequent paragraphs.

SNP, as discussed in section I.4.(v).c., is a compound related to the clinically used nitrovasodilator compounds, and has been used experimentally in many different preparations. Its extensive background in the nitric oxide-related literature combined with its ease of solubility and its polarity made SNP an obvious starting point for iontophoretic experiments with nitric oxide donors.

Against expectations, SNP did not cause an enhancement of responses to somatosensory and EAA stimulation of VB neurones. In contrast, the cells' responses to NMDA were selectively inhibited by SNP applied with a relatively low iontophoretic current, as were the cells' responses to air-jet vibrissal stimulation. SNP therefore appeared to be having a postsynaptic action on NMDA receptors, to inhibit their function. This phenomenon has previously been described in a variety of preparations, including cerebellar granule cells (East *et al*, 1991) and cultured neurones (Manzoni *et al*, 1992a; Kiedrowski *et al*, 1992), and is thought to be independent of nitric oxide production. East *et al* (1991) noted that chemically distinct nitric oxide donors did not produce similar NMDA-inhibitory effects, while ferricyanide which is related to the breakdown products of SNP did mimic SNP's effects. In addition, East *et al* (1991) could observe no difference in SNP's inhibition of NMDA-mediated responses following

the use of the NO scavenger haemoglobin (relative to methaemoglobin, which has a much lower affinity for nitric oxide). Thus it was concluded by a variety of studies that SNP was causing an inhibition of NMDA-mediated responses not by nitric oxide production but via a by-product, probably ferrocyanide ions. These findings (East *et al*, 1991; Manzoni *et al*, 1992a; Kiedrowski *et al*, 1992) were consistent with the effects of SNP seen in the present study.

Subsequent to the generation of the data for the effects of SNP detailed above, another study (Cudeiro *et al*, 1996) applied SNP to a feline dLGN preparation by pressure ejection and found results which contrasted with those of the present investigation, that is an enhancement of visual responses and responses to iontophoretic NMDA application. This may be due to a more effective delivery of nitric oxide by pressure ejection relative to iontophoresis, or to species difference, or as the authors of the study suggested, to differences in the redox state of nitric oxide.

At the very least, the suitability of SNP as a nitric oxide donor in systems involving NMDA receptors had been called into question. Given that alternative nitric oxide donors with fewer complications are available, it would seem sensible to utilise a compound with less possibility of secondary effects, or to design studies to use several chemically distinct nitric oxide donors to ensure that all produce the same pattern of results. In this way, the possibility that what is being observed is not in fact due to nitric oxide production, but to some other factor, can be reduced.

SIN-1 is for many purposes the nitric oxide donor of choice. There are two principal reasons for this. Firstly, the breakdown product of SIN-1 which follows nitric oxide release is thought to be biologically inactive (Manzoni *et al*, 1992b), and so

experiments which utilise SIN-1 are free from the kind of complications which resulted from the use of SNP. Secondly, SIN-1 is relatively stable for a nitric oxide donor (although not as stable as GSNO), being capable of releasing nitric oxide for a period of hours. In addition, SIN-1 is both sufficiently soluble and sufficiently polar to be used in iontophoretic experiments.

SIN-1 produced results which were markedly different from those obtained with SNP; both in VB and in dLGN, SIN-1 caused an enhancement of sensory responses and excitatory amino acid responses of relay neurones. An inhibition of NMDA receptor mediated events such as was observed following SNP ejection onto VB neurones was never seen following the use of SIN-1, and as such the results obtained with this compound were much closer to the properties of a nitric oxide donor which would be predicted from the work of Pape and Mager (1992) and Cudeiro *et al* (1994a&b). The nitric oxide donor which was used in the study of Pape and Mager (1992) in which a depolarising effect was observed was in fact SIN-1.

No difference was observed between the effects of SIN-1 on VB neurones and on dLGN neurones, supporting the suggestion of Jahnsen and Llinas (1984a&b) that thalamocortical relay cells function in a broadly similar manner regardless of the nucleus in which they are located. In the case of the present experiments, visual responses in dLGN were enhanced by iontophoretic SIN-1 ejection in the same way that somatosensory responses were enhanced in VB; responses to iontophoretic application of the excitatory amino acid agonists NMDA and AMPA were also enhanced by SIN-1 in both nuclei.

As discussed above, the breakdown product of SIN-1, SIN-1C, is thought to have no biological action (Manzoni *et al*, 1992b), and as such it was not necessary to

perform controls for its effects. It can be concluded with some certainty, therefore, that the observed effects of SIN-1 in both VB and dLGN were due to nitric oxide production and not due to the effects of another factor. This conclusion was further supported by the observation of the effects of an alternative nitric oxide donor, GSNO, as discussed in the subsequent subsection.

The effects of SIN-1 as described here occurred at iontophoretic ejection currents in the range 10 to 50nA. No additional effects were observed following ejection of the drug with higher currents. This is of potential importance due to possible toxic effects of nitric oxide which may occur at abnormally high concentrations; by ensuring that the measured effects occurred at the lowest possible iontophoretic current, it was possible to reduce the risk of nitric oxide toxicity. However, when deliberately high currents (100-200nA) were applied, no difference in the effect of the drug was seen.

GSNO is unlike SIN-1 is one notable respect, that being its breakdown to release not only nitric oxide but also another potentially biologically active product, glutathione. For this reason it was necessary to perform control experiments for the effects of glutathione to ensure that the observed effects of GSNO were in fact due to nitric oxide production. The outcome of these control experiments proved to be of importance in its own right, and will be discussed in depth in a subsequent subsection, while being discussed in this section where relevant to the effects of GSNO.

Similar to SIN-1, GSNO caused an enhancement of sensory responses (i.e. air-jet vibrissal responses in VB) and of responses to the iontophoretically applied excitatory amino acid agonists NMDA and AMPA. In addition, the effect of GSNO on the response to iontophoretically applied carbachol was also tested; no effect was observed

in this case.

Despite the negative result, the inability of GSNO to potentiate the effects of carbachol on VB neurones is of great importance, as it reveals something of the mechanism of action of GSNO, and therefore of nitric oxide. NOS in the thalamus is known to be located in cholinergic terminals whose origins are in the pedunculopontine and lateral dorsal tegmental nuclei of the brainstem (Bickford *et al*, 1993). The function of these cholinergic neurones is the modulation of the membrane properties of thalamocortical relay neurones, as described in section I.1.(iv).a., in a mechanism involving a combination of nicotinic receptor- and muscarinic receptor-mediated effects. By influencing the potential of relay cell membranes, the cholinergic input to the thalamus is able to bias the firing mode of the cell; thus, acetylcholine's depolarising effect on thalamocortical relay cells tends to shift the membrane potential into the range which favours tonic (rather than burst) firing mode, and favours the faithful relay of ascending sensory information to the cortex. Carbachol, a cholinergic agonist with actions at both nicotinic and muscarinic receptor types, would also tend to depolarise the thalamocortical cell membrane, and is in fact able to cause the cell to fire in the absence of other physiological or pharmacological stimuli. Since NOS is located in the cholinergic terminals in VB and dLGN, it is conceivable that nitric oxide would have a similar function to that of acetylcholine; moreover, Pape and Mager (1992) demonstrated nitric oxide's ability to depolarise thalamocortical cells *in vitro*. A possible explanation for the failure of nitric oxide (released from GSNO) to potentiate the effects of carbachol could, therefore, be found in a common thalamic mechanism of action of nitric oxide and carbachol (or acetylcholine).

As mentioned above, to establish with greater certainty whether GSNO was

acting via nitric oxide production or via its additional breakdown product, glutathione, it was necessary to perform experiments in which glutathione was applied to VB or dLGN neurones by iontophoresis, according to the same experimental protocol as was used for GSNO. These experiments demonstrated that glutathione was in fact inhibitory when applied to thalamic neurones. This piece of evidence is support for the hypothesis that GSNO, which has a facilitatory effect, is acting via nitric oxide production rather than via its glutathione moiety; further support comes from the observations described above of the alternative and chemically distinct nitric oxide donor SIN-1. The possibility remains, however, that GSNO may have actions as a nitrosothiol without complete breakdown to release nitric oxide.

The facilitatory effect of GSNO appeared to be of lesser magnitude than that of SIN-1; while no direct comparison could be drawn from the available data, a hypothesis can nonetheless be proposed for why this may be so. It has been established from the present experiments that GSNO and SIN-1 have facilitatory actions on VB and dLGN neurones, and that glutathione is inhibitory. It follows from this that the effects of GSNO, if it is breaking down to release nitric oxide, would represent a combination of the effects of nitric oxide and the effects of glutathione, with a net facilitatory action which conceivably would be reduced in magnitude compared to that of SIN-1 which has no glutathione moiety (or other inhibitory breakdown product).

D.2.(iii). *Drugs influencing the downstream effects of nitric oxide: 8-Bromo-cGMP and ODQ*

One of the principal means (although far from the only means) by which nitric oxide exerts its effects is via the guanylate cyclase/cGMP system. This takes place

intracellularly, such that nitric oxide stimulates the enzyme soluble guanylate cyclase to increase the rate of production of the cyclic nucleotide second messenger cGMP.

Unfortunately, it was not possible using the protocol of the present experiments to use ODQ, the recently developed inhibitor of soluble guanylate cyclase. However, interesting results were obtained using the membrane permeant analogue of cGMP, 8-Br-cGMP.

8-Br-cGMP produced a set of results which were strikingly similar to those obtained using nitric oxide donors, i.e. an enhancement of responses to sensory stimuli and to the excitatory amino acid agonists NMDA and AMPA, both in dLGN and in VB. While this in itself does not provide evidence that nitric oxide in the thalamus acts via the guanylate cyclase/cGMP system, it does lend support to the hypothesis that nitric oxide is, at least in part, acting via this system rather than via a direct effect on excitatory amino acid receptors.

The subsequent effects of cGMP (or 8-Br-cGMP) are less clear, but may involve actions on cation conductances (Ahmad *et al*, 1994; Kurenny *et al*, 1994) or on cGMP dependent protein kinases (Lincoln and Cornwell, 1993) which are known to be present in the thalamus (El Husseini *et al*, 1996).

Pape and Mager (1992) found that the effects of the nitric oxide donor SIN-1 in the thalamus were mimicked and occluded by coapplication of 8-Br-cGMP. This is stronger evidence for the transduction of nitric oxide's effects being via this biochemical pathway. However, Cudeiro *et al* (1994b) did not observe similar effects, in experiments in which iontophoretically applied 8-Br-cGMP failed to reverse the effects of a nitric oxide synthase inhibitor. Following this result, the authors concluded that nitric oxide was probably not acting via the guanylate cyclase/cGMP system, a conclusion which

appears to be contradictory to the present results and to those of Pape and Mager (1992). The explanation for this may lie in species difference, or in a difference in the manner in which the 8-Br-cGMP solution was prepared for iontophoresis; Cudeiro *et al* (1994b) used a 10mM solution of 8-Br-cGMP at pH 4.5, and as such were presumably ejecting the drug as a positive ion. The present experiments, by contrast, prepared 8-Br-cGMP as a basic solution (pH 8) at 50mM and ejected it as a negative ion; it may be the case that the drug is ejected more efficiently in this way.

D.3. GLUTATHIONE

Although initially intended as control experiments for the possible effects of glutathione resulting from the breakdown of GSNO, experiments involving glutathione proved to be of interest and importance in their own right.

When applied to VB neurones by iontophoresis, the reduced form of glutathione caused an inhibition of sensory and excitatory amino acid responses. Similar results were found using GSH in dLGN, and using the oxidised form of glutathione (GSSG) in VB. As well as being important evidence for the facilitatory action of GSNO being mediated by nitric oxide, this observation suggests a possible modulatory role for glutathione in the thalamus.

Glutathione (see section I.3.) is known to be present at high concentrations throughout the central nervous system (Slivka *et al*, 1987a&b), being especially abundant in glial cells (Raps *et al*, 1989). However, Zängerle *et al* (1992) crucially reported the release of glutathione from neuronal cells, which lends support to the suggestion that glutathione may have a neurotransmitter role, rather than merely a role as an antioxidant.

The effects of glutathione on neurotransmission as described in the literature are highly varied. The presence of binding sites on astrocytes has been revealed using immunostaining (Guo and Shaw, 1992; Guo *et al*, 1992) while other studies (Ogita *et al*, 1986; Ogita and Yoneda, 1988) have demonstrated a glutathione-induced displacement of glutamate from its binding sites. Moreover, direct interactions with excitatory amino acid neurotransmission have been reported (Varga *et al*, 1989; Ogita *et al*, 1995) as has glutathione's ability to influence the redox state of the NMDA receptor (Janáky *et al*, 1993). More recent work has shown a direct stimulation of Na^+ currents by glutathione in cortical neurones, and a hypothesis has been proposed in which

glutathione could be activating its own receptor/ionophore (Pasqualotto *et al*, 1996; Shaw *et al*, 1996).

The suggestion of an excitatory action for glutathione in the cortex is unlike the effects observed in the present study in the thalamus, which indicate that glutathione has a negative modulatory role in this brain region. In the case of the experiments with GSH, it appears that the inhibitory effect is not mediated by an effect at the NMDA receptor, as AMPA responses were also inhibited. Other studies (Levy *et al*, 1991) have reported a neuroprotective role for glutathione in NMDA receptor-mediated excitotoxicity, which would be in line with the present results; similarly, Gilbert *et al* (1991) have observed an inhibitory effect of GSSG on NMDA responses in cultured neurones.

CHAPTER 5

CONCLUSIONS

C.1. FULFILMENT OF THE AIMS OF THE PRESENT RESEARCH AND FUTURE WORK

In section I.5 (the aims of the present research) a list of hypotheses was proposed. These, along with suggestions of how the work could be taken further, are addressed as follows:

(i) *Nitric oxide donors when iontophoretically applied should have an excitatory action on VB or dLGN neurones.*

This was the case, with no difference being observed between VB and dLGN. The excitatory action encompassed effects on not only sensory responses, but also on responses to excitatory amino acids, but not to the cholinergic agonist carbachol.

(ii) *Chemically dissimilar NO-donors should produce similar effects if their action is mediated by NO.*

GSNO and SIN-1 are chemically unrelated to each other and produced the same profile of effects, providing evidence that both were acting via nitric oxide production. SNP produced anomalous effects, which can be explained in terms of the effects of an unwanted by-product. Use of other chemically dissimilar NO donors, and possibly also NO itself, should further emphasise that the observed response is mediated by NO and not by a by product of donor breakdown.

(iii) *Nitric oxide synthase inhibitors should conversely be inhibitory when applied to VB or dLGN neurones.*

L-NAME failed to produce the inhibition suggested by this hypothesis; further hypotheses to explain why this should be so have been proposed. Clearly, one line of further investigation (for dLGN cells) would be to test the NOS inhibitors against the potentiating effects of L-arginine. In addition, the use of inhibitors selective for the neuronal isoform of NOS, such as &-NI and TRIM, may produce more reliable results.

(iv) *The breakdown products of NO-donor compounds should not replicate the effects of the donor itself (e.g. glutathione should not mimic the effects of GSNO).*

Glutathione did not replicate the effects of GSNO, providing evidence for GSNO acting via nitric oxide production. Glutathione did, however, have important effects in its own right.

The identification of glutathione as having interactions with excitatory amino acid neurotransmission in the thalamus opens up several important avenues of research: firstly, it would be of interest to use immunohistochemical techniques to attempt to identify whether endogenous glutathione is present in the thalamus; secondly, the sites of storage and release of glutathione could be localised, using similar techniques; thirdly, it has been suggested (Pasquallotto *et al*, 1996; Shaw *et al*, 1996) that glutathione has its own binding sites, possibly even its own receptors, and so identification of such binding sites in the thalamus could have important implications for the elucidation of the mode of action of this compound.

(v) *L-arginine when applied to dLGN cells should have a facilitatory action, as has been previously observed in VB.*

L-arginine did indeed have a facilitatory action in dLGN. It has ben suggested (Do *et al*, 1994) that L-arginine is released from glial cells in the thalamus as a means of initiating NO synthesis in the terminals of cholinergic terminals from the brainstem. The trigger for L-arginine's release, however, remains unclear, and as such it would be useful to test compounds (for example, antagonists at metabotropic glutamate receptors), applied through a push-pull perfusion cannula, in order to establish whether the test compounds are capable of either stimulating or inhibiting the L-arginine release which follows sensory stimulation.

(vi) *If thalamic nitric oxide exerts its action via the guanylate cyclase/cyclic GMP system, it should be possible to replicate the effects of increasing or decreasing the NO concentration by pharmacologically manipulating the level of cyclic GMP in relay neurones.*

8-Br-cGMP, by mimicking the effects of increasing the concentration of cGMP and therefore mimicking one of the possible effects of nitric oxide produced similar results to nitric oxide donors, providing evidence in support of the suggestion that nitric oxide is acting via this system. It was not possible, however, to inhibit soluble guanylate cyclase with ODQ to decrease the concentration of cGMP intracellularly. An important line of research would be to apply ODQ by pressure ejection, to overcome the problems encountered with its iontophoresis, and so to establish whether nitric oxide in the thalamus acts via the guanylate cyclase/cGMP system, or by some other means.

C.2. FINAL CONCLUSIONS

The work detailed in this thesis provide further evidence for the involvement of nitric oxide in the function of the thalamus; this role appears to be facilitatory, allowing the faithful passage of sensory information ascending to the cortex. Nitric oxide may act in a similar manner to acetylcholine, and may utilise the same mechanism of action at the biophysical level. As such, nitric oxide an important role for nitric oxide is suggested in the modulation of arousal and attentiveness which occurs in the thalamus.

In addition, the work has been detailed which for the first time demonstrates an inhibitory effect of glutathione in the thalamus. This effect may represent a modulatory effect of this compound.

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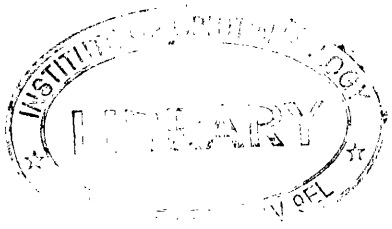

APPENDIX 1

ABBREVIATIONS

(1S,3R)-ACPD	(1S,3R)-aminocyclopentane-1,3-dicarboxylic acid
AHP	after-hyperpolarisation
AMPA	α-amino-4-hydroxy-5-methyl-4-isoxazole propionic acid
AP4	L-2-amino-4-phosphonobutanoate
AP5	D-2-aminophosphonopentanoic acid
cAMP	cyclic adenosine 3',5'-monophosphate
BH_4	tetrahydrobiopterin
8-Br-cGMP	8-bromoguanosine 3'5'-cyclic monophosphate
L-CCG-I	2S,1'S,2'S-2-(2'-carboxycyclopropyl)glycine
cGMP	cyclic guanosine 3',5'-monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
S-4-CPG	(S)-4-carboxyphenylglycine
CPP	3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate
dLGN	dorsal lateral geniculate nucleus
DTNB	5,5-dithio-bis-2-nitrobenzoic acid
DTT	dithiothreitol
EAA	excitatory amino acid
EDRF	endothelium derived relaxing factor
EEG	electroencephalogram
eNOS	endothelial nitric oxide synthase
EPSP	excitatory postsynaptic potential

FAD	flavin adenine dinucleotide
FK-409	(±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide
FMN	flavin mononucleotide
GABA	gamma amino butyric acid
GAD	glutamic acid decarboxylase
GKI	cGMP-dependent protein kinase type I
GKII	cGMP-dependent proetin kinase type II
G-protein	guanyl nucleotide binding protein
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	oxidised glutathione (glutathione disulphide)
HCA	homocysteic acid
HRP	horseradish peroxidase
5-HT	5-hydroxytryptamine
IBMX	isobutylmethylxanthine
iNOS	inducible nitric oxide synthase
IPSP	inhibitory postsynaptic potential
LTP	long term potentiation
LTS	low threshold spike
L-MeArg	N^G-monomethyl-L-arginine
mGluR	metabotropic glutamate receptor
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAAG	N-acetylaspartylglutamate
NADPH	nicotinamide adenine dinucleotide phosphate

	(reduced form)
NADPH-diaphorase	reduced nicotinamide adenine dinucleotide phosphate diaphorase
L-NAME	L-arginine methylester
NBQX	2,3,-dihydroxy-6-nitro-7-sulphamoyl-benzo(f) quinoxaline
7-NI	7-nitroindazole
7-NINa	7-nitroindazole (sodium salt)
NMDA	N-methyl D-aspartate
L-NNA	N^G-nitro-L-arginine
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NONOate	1-substituted diazan-1-ium-1,2-diolate
NOS	nitric oxide synthase
ODQ	1H-[1,2,4]-oxadiazolo-[4,4,a]-quinoxalin-1-one
PGN	perigeniculate nucleus
PSTH	peristimulus time histogram
RNA	ribonucleic acid
SC	superior colliculus
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
SNOC	S-nitrosocysteine
SNOG	S-nitrosoglutathione

TRN	thalamic reticular nucleus
VB	ventrobasal complex of the thalamus
VPL	ventral posterolateral nucleus
VPM	ventral posteromedial nucleus