ISCHEMIA-INDUCED AMNESIA IN RATS: A NEUROBEHAVIOURAL ANALYSIS

by

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Abstract

It is well known that transient cerebral ischemia can result in irreversible neuronal damage and permanent learning and memory impairments in humans. The primary objective of this thesis was to develop a rat model of ischemia-induced amnesia that can be used both to investigate the neuropathological basis of ischemia-induced memory impairments, and to assess the ability of potential anti-ischemic drugs to prevent these impairments.

First, quantitative histological techniques were used to assess the extent and location of neuronal damage produced by a bilateral carotid occlusion method of ischemia induction in rats. The neuropathological changes observed were similar to those typically associated with ischemia-induced amnesia in humans; the predominant lesion involved hippocampal CA1 pyramidal neurons, and there was no detectable extrahippocampal damage. From these results we concluded that this method of inducing ischemia is appropriate as a component of a rat model of ischemia-induced amnesia.

The experiments in Chapter 3 investigated the effects of ischemia-induced brain damage in rats on their performance of nonspatial memory tasks analogous to tasks on which amnesic patients show impairments. In Experiment 3.1, rats were tested on a one-trial object recognition task that is based on the spontaneous differential exploration of novel and familiar objects. Sham-ischemia control rats spent more time investigating a novel object than a familiar object, whereas ischemic rats spent the same amount of time investigating each of the two objects. The performance of ischemic rats is consistent with a recognition memory impairment, although it may reflect a change in
Abstract

novelty preference. In Experiments 3.2 and 3.3, rats were tested on a delayed nonmatching-to-sample (DNMS) task that was designed to mimic the DNMS task on which monkeys and humans show deficits following ischemia. Ischemic rats were significantly impaired on DNMS compared to sham-ischemia control rats, both in learning the task and in their subsequent test performance at retention delays of 4, 15, 30, 60, 120, and 300 s. Furthermore, extensive presurgery training on DNMS did not reduce this impairment. The performance of ischemic rats on DNMS was similar to that previously reported for monkeys and humans with ischemic brain damage, indicating that ischemia-induced memory impairments are similar in rats, monkeys and humans.

The experiments in Chapter 4 used the rat model of ischemia-induced amnesia to assess the therapeutic potential of a glycine receptor antagonist, both at an anatomical level, and at a behavioural level. The quantitative histological analyses performed in Experiment 4.1 showed that 7-Chlorokynurenic acid, administered immediately prior to ischemia, significantly reduced ischemic CA1 cell loss. The preliminary behavioural analyses in Experiment 4.2 indicated that 7-Chlorokynurenic acid also attenuates the ischemia-induced DNMS impairment.

On the basis of these findings, it appears that this rat model of ischemia-induced amnesia may be useful, both in the investigation of the neuropathological basis of ischemia-induced amnesia, and in the behavioural assessment of neuroprotection in ischemia.
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CHAPTER 1: GENERAL INTRODUCTION

Various clinical conditions including cardiac arrest, severe hypotension, seizure activity and atherosclerosis of the carotid or vertebral arteries can result in cerebral ischemia (Dearden, 1985). Cerebral ischemia refers to a reduction in cerebral circulation to levels that are insufficient to meet metabolic demands. Such reductions in the blood supply to the brain can cause permanent neuronal damage (Fields and Lemak 1989). Due to advances in medical care there has been a dramatic increase in the number of survivors of ischemic episodes (up to 200,000 patients per year in the USA survive stroke alone (American Heart Association, 1986)). This has led to increasingly wide interest in the neurological consequences of cerebral ischemia. It is estimated that at least 20% of cardiac arrest survivors have cognitive deficits (Davis & Volpe, 1990), and impairments of learning and memory have been recognised as one of the most common permanent cognitive disabilities (Volpe and Petito 1985).

Over recent years considerable research has been aimed towards an understanding of the mechanisms of ischemia-induced brain-damage, and towards the development of pharmacological agents that might protect patients suffering ischemic episodes against both the brain damage and the subsequent cognitive impairments. The development of several animal models of ischemia has aided this endeavour, such that now the cellular and molecular mechanisms of ischemic brain damage are fairly well understood, and several drugs have been shown to impart partial protection against the damage in animals. However, relatively little research has been directed towards characterising the impairments in learning and memory that result from ischemia, and investigating the specific neuropathological changes underlying these impairments. The development of animal models of ischemia-induced amnesia is vital to the analysis of
Chapter 1

the functional consequences of ischemic brain damage, and also to testing the
behavioural efficacy of potentially therapeutic drugs.

This thesis has two objectives. The first is to describe a rat model of ischemia-
induced amnesia that is comparable to the amnesic syndrome produced by ischemia in
humans. The second is to use this model as a functional assay against which the
efficacy of a potentially protective drug can be tested. Accordingly, the General
Introduction of this thesis is divided into 5 sections. The first section describes
ischemia-induced amnesia and the underlying brain damage in the clinical population.
The second section provides an account of descriptions of learning and memory deficits
in animals following ischemia, and the patterns of damage that are associated with
them. The third section includes an overview of the cellular and molecular mechanisms
of ischemic brain damage, concentrating mainly on the preeminent "excitotoxic theory"
of ischemia. The fourth section describes the protective effects offered by drugs that
interfere with the excitotoxic actions of glutamate. The final section is a summary of
the research described in this thesis.

ISCHEMIA-INDUCED AMNESIA IN HUMANS

Amnesia is defined as a pure memory impairment in the absence of other
primary symptoms (Baddeley, 1990). There have been several reports of cases of
amnesia resulting from transient cerebral ischemia (e.g. McNeill, Tidmarsh, & Rastall,
1965; Muramoto, Kuru, Sugishita, & Toyokura, 1979, Finklestein & Caronna, 1978),
but only in the last decade has there been a systematic characterisation of the amnesic
syndrome following ischemia (Volpe & Hirst, 1983; Graf, Shimamura & Squire, 1985;
Zola-Morgan, Squire, & Amaral, 1986; Squire & Shimamura, 1986; Squire, Zola-
Morgan, & Chen, 1988). Post-mortem neuropathological analyses of the brains of amnesic patients who survived the primary ischemic episode have been conducted in only very few cases (Cummings, Tomiyasu, Read, & Benson, 1984; Zola-Morgan et al., 1986). The most detailed of these descriptions is that of the patient R.B. who, at the age of 52, suffered a severe ischemic episode following coronary bypass surgery. R.B.’s neuropsychological profile was catalogued over the five years between his initial ischemic episode and his death (Zola-Morgan et al., 1986; Graf, Squire, & Mandler, 1984; Graf et al., 1985) and, following his death, a detailed neuropathological analysis of the brain damage induced by ischemia was performed. This case study thus represents a rare opportunity to describe the relationship between ischemia-induced amnesia and the underlying brain damage. The following sections include a description of the neuropsychological and neuropathological sequelae of transient ischemia illustrated by the case R.B., and a discussion of the features of ischemia-induced amnesia that are pertinent to this thesis.

The case of R.B.

Neuropsychological Profile

Six months following his ischemic episode, R.B. was given a battery of standard neuropsychological tests. These tests indicated that while he appeared to have above average intelligence (as measured by the full scale Wechsler Adult Intelligence Scale (WAIS)), he was impaired on several tests of memory, and his score on the Wechsler Memory Scale (WMS) was 20 points lower than his WAIS score. The difference between the WAIS and WMS scores provides a fairly reliable index of the
severity of memory impairment (although see Squire & Shimamura 1986), a difference of 20 usually being classified as a substantial impairment.

After this initial assessment, and over the next five years until his death, R.B. was tested on a number of formal tests that have been shown to be useful for characterising amnesic patients (Squire & Shimamura 1986). These tests can be divided into three categories: 1. those examining new learning ability (to assess anterograde amnesia); 2. those examining memory for events that occurred prior to the ischemic episode (to assess the extent and time course of retrograde amnesia); and 3. tests of cognitive function other than memory. In addition, R.B.'s performance was evaluated on a set of memory tests on which patients with severe amnesia show no impairment (Graf et al., 1985). R.B.'s performance on tests in each of these four categories will be described in turn.

1. R.B. was severely impaired on three tests of new learning ability (paired associate learning, story recall and diagram recall) administered 6, 9 and 23 months after his ischemic episode. In paired associate learning, subjects are presented 10 unrelated word pairs on each of three study trials. After each study trial, the subject is shown the first word of each pair and asked to recall the second word. R.B.'s mean score over the three sessions was 3.7 out of a total of 30. For story recall, subjects are read a short prose passage containing 21 segments, and asked to recall it immediately after hearing it and again, 12 min after presentation. R.B. could recall 5 segments immediately, and an average of only 0.7 segments after the delay. For the diagram recall test, subjects are asked to copy a complex figure, and then to reproduce it from memory after a 12 min delay. R.B. averaged 29 points out of a possible 36 for his copy, and 3 points for his reproduction. R.B.'s scores on these tests were comparable
to those of other amnesic patients, including three other patients with ischemia-induced amnesia (Squire & Shimamura 1986), and revealed severe anterograde amnesia for both verbal and nonverbal information. R.B. was also impaired on tests of free word recall and recognition administered throughout the five years following his ischemic episode. However, he was unimpaired on tests of immediate memory and he had normal digit span.

2. In contrast to his severe anterograde amnesia, R.B. showed little impairment on tests of memory for premorbid events. The tests used included recall and multiple choice recognition of news events that had occurred in one of the 4 decades prior to his ischemic episode, the Boston Famous Faces Test which requires the identification of photographs of people who became famous at various times prior to his ischemic episode, multiple choice recognition tests for television programmes that were broadcast for a single season, and a test of autobiographical recall. R.B.'s performance on each of these tests was normal, with the exception of a slight impairment for news events and television programmes in the three years prior to his ischemia. These results suggest that R.B. may have had some retrograde amnesia limited to these three years.

3. R.B. performed normally on two tests known to be sensitive to cognitive dysfunction, the Boston Naming Test, which assesses the ability to name drawings of objects, and the Dementia Rating Scale. Together with an above average score on the WAIS, which measures general intellectual capacity, these tests demonstrate the selectivity of R.B.'s memory impairment.

4. R.B. performed normally on priming tasks (Graf et al., 1984, 1985). Priming refers to the facilitative effects of a prior experience on performance of a task, and may occur in the absence of conscious recollection of the learning experience. For
example, in one priming task, subjects were exposed to a list of 15 words and asked to rate them on a 5-point scale in terms of whether they liked or disliked the words. Some time following this study phase, the subjects were given a word-completion task in which they were asked to complete word beginings (i.e. the first few letters of a word) with the first word that came to mind. Ten of the word beginings could be completed with words on the study list, and ten could not. In this, and similar tasks, control subjects produced the recently presented words as completions for the test cues more than four times as often as would be expected if the words had not been presented previously. This facilitative phenomenon is known as priming (Graf & Schacter, 1985). R.B., showed intact priming on word-completion tasks and a word categorisation task, despite a severe impairment on a free-recall test and a word recognition test of the same study material (Graf et al., 1985).

Summary and comparison with other examples of ischemia-induced amnesia

R.B. exhibited severe anterograde amnesia, demonstrated by his poor performance on tests of new learning ability, and he may also have suffered some retrograde amnesia limited to the few years immediately prior to his ischemic episode. These deficits occurred in the absence of any other cognitive impairments. R.B.’s anterograde amnesia was characterised by long term memory deficits, with intact immediate memory, and it was global; it encompassed information in all sensory modalities tested, and included both verbal and nonverbal information. His memory deficits were particularly striking in tasks in which a delay was interposed between the study and test phases. Despite his severe anterograde amnesia, R.B. was capable of learning in some situations, as shown by intact priming.
The neuropsychological profile described for R.B. is similar to that described for other patients with ischemia-induced amnesia, who also do badly on tasks of new learning ability such as paired associate learning, story recall and diagram recall (Squire & Shimamura, 1986). These patients, like R.B., are also more impaired on recognition tasks that incorporate a delay than on tests of immediate recognition. Most patients with ischemia-induced amnesia do not display any retrograde amnesia, and those that do usually have amnesia only for events that occurred over the period of time leading up to the ischemic event, and not for events in the distant past (Volpe & Hirst, 1983). However, more subtle or temporally limited retrograde amnesia may occur, and not be detected with the tests commonly used (Zola-Morgan et al., 1986). Finally, other ischemic patients tested also show intact priming (Graf et al., 1985).

Neuropathological Profile

After his death, a detailed histological examination of R.B.'s brain was conducted. This revealed a bilateral lesion involving the entire mediolateral and rostrocaudal extent of the CA1 field of the hippocampus. Minor pathology was observed in the right postcentral gyrus, the left globus pallidus, and the left internal medullary lamina of the thalamus, and small foci of cell loss were observed in the anterior amygdaloid area, the left internal capsule, the neocortex, and the cerebellum. However, these minor abnormalities were not very extensive. The rest of the hippocampus was unaffected except for minor cell loss in the CA3 and subiculum identified in two sections.

The neuropathological profile in the case of R.B. is representative of the general pattern of brain damage seen following transient global ischemic insults clinically.
(Adams, Brierley, Connor, & Treip, 1966; Graham, 1977). The most prominent feature of the brain damage that occurs in such cases is that despite a global insult, only specific brain regions, such as the hippocampus, are affected. The observation that specific brain regions may be more vulnerable to ischemic injury than others was first made at the beginning of this century (Spielmeyer, 1925, Vogt & Vogt, 1937). More recently, the pattern of selective vulnerability to ischemia has been documented in detail, and it has transpired that rather than specific brain regions being vulnerable to ischemia, specific cells are vulnerable. First, different cell types show differential selectivity; neurons are much more sensitive to ischemia than oligodendrocytes, astrocytes and endothelial cells, which in turn exhibit decreasing vulnerability (Jacob, 1963). Among neurons, those within the most recently evolved brain structures are among the most vulnerable (Graham, 1977). Specifically, examination of the human brain has shown that there is a hierarchy of neuronal susceptibility to ischemia as shown in Table 1 (Pulsinelli, 1985; Brierley, 1977). The CA1 pyramidal neurons in the hippocampal formation are among the most vulnerable cells in the brain.

The neuropathological profile of R.B. concurs with this hierarchy; the primary lesion was of CA1, and within the hippocampal formation there was also extensive loss of CA2 neurons while most of the CA3 and dentate granule cells were spared. Also consistent with the hierarchy was patchy loss of Purkinje cells in the cerebellum and scattered cell loss in the neocortex. The other extrahippocampal lesions appeared to be restricted to small foci, and may have been caused by small strokes induced by atherosclerosis of cerebral vessels, although there is no direct evidence for this.
Table 1 Order of Neuronal Vulnerability to Transient Forebrain Ischemia (adapted from Pulsinelli, 1985).
<table>
<thead>
<tr>
<th>Order of Neuronal Vulnerability</th>
<th>HIPPOCAMPUS</th>
<th>CEREBELLUM</th>
<th>STRIATUM</th>
<th>NEOCORTEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal</td>
<td>CA1, Hilar neurons</td>
<td>Purkinje cells</td>
<td>Small and medium cells</td>
<td>Layers 3, 5 &amp; 6</td>
</tr>
<tr>
<td>Ventral</td>
<td>CA4</td>
<td>Stellate and basket cells</td>
<td>Large cells</td>
<td>Layers 2 &amp; 4</td>
</tr>
<tr>
<td></td>
<td>CA3</td>
<td>Granule Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dentate granule cells</td>
<td>Golgi cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Chapter 7
Two features of ischemia-induced amnesia that are illustrated by the case of R.B. are particularly relevant to the development of animal models of ischemia-induced amnesia. The first is that patients with ischemia-induced amnesia display long-term memory impairments in some situations but not in others, as shown by intact priming, and normal learning and retention of a variety of motor, perceptual and cognitive skills. The second is that studies which combine neuropathological and behavioural analyses allow investigation and speculation as to the anatomical basis of ischemia-induced amnesia. These two features, and their importance to animal models, will be discussed in turn.

The finding that patients with ischemia-induced amnesia, in common with amnesic patients with a variety of etiologies (Squire, 1987), display memory impairments in some situations but not in others implies that there are multiple kinds of long-term memory, at least one of which is impaired in amnesia and at least one of which is not. The notion that there are multiple distinguishable types of long-term memory, suggests in turn that there are multiple anatomically distinct memory systems.

Several theoretical schemes have been proposed to describe these multiple memory systems. These descriptions are often expressed as dichotomies, and distinguish between the long term abilities that are impaired in amnesia and those that are spared. One such scheme distinguishes between explicit and implicit memory (Graf & Schacter, 1985). Explicit memory refers to the conscious recollection of previous experiences at the time of retrieval, whereas implicit memory refers to the retrieval and expression of information stored from previous experiences in the absence of conscious recollection. Patients with ischemia-induced amnesia are impaired on explicit memory.
tasks (e.g. recognition), but they exhibit normal or near normal performance on implicit memory tasks (e.g. priming).

Another theoretical scheme, that distinguishes between the memory abilities that are impaired in amnesia and those that are spared, postulates separate systems for declarative memory and procedural memory. Declarative memory, which is impaired in amnesia, is memory for facts and data that are acquired through learning, and is accessible to conscious recollection. It is thought to be based on neural representations of previously experienced perceptions, thoughts, or facts that can be described verbally or declared (Squire, 1986). Procedural memory, which is spared in amnesia is inherent in the performance of skilled actions and revealed by changes in the quality of those actions. Procedural or nondeclarative memory includes skill learning, priming effects, dispositions to respond to stimuli, for example as a result of conditioning, and nonassociative learning (Zola-Morgan & Squire, 1990). Declarative memory is thought to represent one memory system, whereas nondeclarative memory may be comprised of multiple systems, none of which are affected in amnesia.

Other dichotomies that have been applied to the dissociation between lost and spared memory abilities in amnesia, and which are conceptually similar to the declarative-procedural distinction include "memory" versus "habit" (Mishkin, Malamut, & Bachevalier, 1984), "representational memory" versus "dispositional memory" (Thomas & Gash, 1988) and "conscious recollection" versus "automatic recollection" (Baddeley, 1982). Other distinctions that are used frequently to dissociate between different types of long term memory, but which do not map so well on to the declarative-nondeclarative memory distinction, include "episodic memory" versus "semantic memory" (Schacter & Tulving, 1982), and "working memory" versus
"reference memory" (Honig, 1978). The working memory-reference memory distinction will be discussed in more detail below, as it is used commonly to describe the features of tasks designed for animals.

The relevant issue to animal models of ischemia-induced amnesia is that it is essential to use tasks which tap the memory abilities that are lost in amnesia. It is therefore important to analyse the tasks used, and to compare the performance of animals and humans keeping in mind the dissociations between memory systems as described above.

The combined neuropsychological and neuropathological analyses in the case of R.B. allow us to speculate as to the anatomical basis of his amnesic syndrome. Given the pattern of cell loss commonly seen in ischemia, it has been proposed that cell loss within the hippocampus is responsible for ischemia-induced memory deficits in humans (Cummings et al, 1984; Zola-Morgan et al., 1986), and certainly the case of R.B. supports this idea. This proposal is also supported by the neuropsychological data from patients who are amnesic following medial temporal lobe lesions (e.g. Scoville & Milner, 1957; Victor, Angevine, Mancall, & Fischer, 1961). These patients have large hippocampal lesions, although they also sustain damage to other medial temporal lobe structures including the amygdala, the temporal stem, and surrounding cortical areas. Recent experiments with monkeys suggest that damage to these extrahippocampal structures may be responsible for the medial temporal lobe amnesic syndrome (e.g. Mishkin, 1978; Murray & Mishkin, 1984; Cirillo, Horel & George, 1989; Zola-Morgan et al, 1989). The role of damage restricted to the hippocampus in amnesia is therefore uncertain.
In summary, synthesis of the neuropathological and neuropsychological data from ischemic patients leads to the conclusion that the hippocampus is required for declarative, or explicit memory, but not for procedural, or implicit memory. However, given the sparse clinical studies examining the neuropsychological and neuropathological consequences of ischemia, the problems with post-mortem histological studies in humans, and common reports of extrahippocampal damage in amnesic patients (both ischemic and non-ischemic) it is necessary to turn to animal models of ischemia-induced amnesia for insight into the neuropathological basis of ischemia-induced amnesia.

**ANIMAL MODELS OF ISCHEMIA-INDUCED AMNESIA**

The rationale behind the development of animal models of ischemia-induced amnesia is first to assess whether ischemic brain damage results in deficits of learning and memory analogous to those seen in humans and, if this is the case, to evaluate the neuropathological changes which mediate these deficits. A vital first step, therefore, is the development of animal models of ischemic brain damage. In particular, it is important to ensure that ischemic brain damage in animals reflects the pattern of selective vulnerability found in humans. The second stage of the process involves assessing the learning and memory abilities of animals on tasks that reflect the situations in which patients with amnesia are impaired, i.e., to use tests of declarative or explicit memory rather than of procedural or implicit memory. Clearly, the more analogous the tasks are to tests used in the clinical situation, the easier it will be to compare the performance of humans and animals.
When developing animal models of ischemia-induced amnesia, it is necessary to assume that a certain pattern of neuronal damage will result in similar behavioural deficits across species. However, this may not always be the case as there are many anatomical differences between the brains of mammalian species, and it is possible that different brain structures or systems mediate certain behaviours or abilities in different species. The results from animal models of ischemia-induced brain damage must therefore be interpreted with possible differences in mind.

In this section I will first describe the experimental animal models of ischemic brain damage which have been utilised in the development of animal models of ischemia-induced amnesia. I will then describe, in turn, the monkey models and rat models of ischemia induced amnesia that have been developed. For each of these models I will describe the behavioural findings and their correspondence to the clinical reports of ischemia-induced amnesia, and discuss the relationship between the behavioural and neuropathological consequences of cerebral ischemia.

**Animal models of brain damage induced by transient global ischemia**

Two types of ischemia predominate in clinical medicine: global ischemia, which is associated most commonly with cardiac arrest, and focal ischemia, which is caused by stroke (Ginsberg & Busto, 1989). In global ischemia, the blood supply to the whole brain is compromised, and the ischemia is extensive. Moreover, the period of ischemia must be relatively brief to allow survival. In contrast, focal ischemia affects only a part of the brain, and may be caused by transient or permanent occlusion of an artery. The ischemia is usually severe only in part of the tissue, and this area is surrounded by a penumbra of tissue which is rendered partially ischemic. The following discussion will
deal only with animal models that have been developed to investigate transient global ischemia, as the focus of this thesis is the amnesic syndrome and pathological changes that are induced by global ischemia.

Global ischemia can be induced in rats by cardiac arrest (Blomqvist & Weiloch, 1985). However, in most experiments global ischemia is mimicked by temporary occlusion of the large extracranial blood vessels that supply the brain. The brain is supplied by two carotid arteries and two vertebral arteries which, in most species, are connected at the base of the brain in the Circle of Willis. At the Circle of Willis these arteries divide into the cerebral arteries which supply different areas of the brain. Transient occlusion of these large extracranial vessels therefore induces severe transient forebrain ischemia which simulates the global circulatory disturbances that occur during cardiac arrest. However, the blood supply to the brain stem is still intact, allowing recovery without artificial ventilation or intensive care (Siesjo & Smith, 1991).

Severe transient forebrain ischemia can be achieved in rats either by four vessel occlusion (4VO), which involves permanent bilateral occlusion of the vertebral arteries combined with transient bilateral occlusion of the carotid arteries (Pulsinelli & Brierley, 1979) or by a two vessel occlusion method (2VO) which combines transient bilateral carotid occlusion and systemic hypotension (Smith, Bendek, Dahlgram, Rosen, Wieloch, & Siesjo, 1984). In Mongolian gerbils transient bilateral carotid occlusion without hypotension is commonly used to induce severe forebrain ischemia (Kirino, Tamura, & Sano, 1985). Hypotension is not necessary as approximately 50 percent of this species lacks communication between the carotid and vertebral arteries (Berry, Wisniewski, Svarzbein, & Baez, 1975).
These experimental animal models of ischemia result in selective neuronal necrosis, similar to that described in humans (i.e. neurons are damaged whereas glia and vascular cells remain intact) (Schmidt-Kastner & Freund, 1991). Furthermore, the pattern of selective vulnerability of neurons to transient global or forebrain ischemia is similar across species and models of ischemia (Schmidt-Kastner & Freund, 1991, Pulsinelli, 1985; Brown, 1977). For example, pyramidal neurons in the CA1 field of the hippocampus and somatostatin-containing neurons in the hilus of the dentate gyrus are the most susceptible neurons in each of the models (Petito & Pulsinelli, 1984; Auer, Jensen, & Whishaw, 1989; Kirino & Sano, 1984). In general, the extent and location of damage is dictated by the duration of the ischemic insult. For example, in the 4VO model in rats, as the duration of bilateral carotid occlusion is increased from 10, through 20, to 30 min, the extent of CA1 cell loss increases, and in the 20 and 30 min occlusion procedures loss of small and medium sized neurons in the striatum and of neurons in layers 3, 5 and 6 of both anterior and posterior neocortex is also detected. CA3 neurons are seldom damaged (Petito & Pulsinelli, 1979; Johansen, Zimmer, & Diemer, 1987; Johansen, Jorgensen, von Lubitz, & Diemer, 1984; Jorgensen & Diemer, 1982; Kirino, Tamura, & Sano, 1985). With 2VO combined with systemic hypotension in rats, brief periods of ischemia result in the loss of CA1 and hilar neurons, whereas following longer insults extrahippocampal damage is seen in the dorsolateral caudate and neocortex, and with even longer periods of ischemia, in the amygdala, reticular nucleus of the thalamus, septal nuclei, substantia nigra and the cerebellar vermis (Smith, Auer & Siesjo, 1984). In the monkey, 12-14 min of global ischemia induced by inflation of a neck cuff results in damage restricted to CA1 pyramidal neurons and hilar neurons (Miller, & Myers, 1972). Similarly in gerbils
subjected to 2VO, occlusion for 5 min results in CA1 pyramidal cell loss, whereas
damage in CA3 is detected only if the occlusion lasts for 20 or 30 min (Kirino et al.,
1985).

These animal models of ischemia have reproduced the finding in humans of
selective vulnerability to ischemia of particular neurons. For example, in all species,
the pyramidal neurons in CA1 are exquisitely vulnerable, whereas those in CA3 are
relatively resistant. In addition, they have provided important information regarding the
time course of ischemic brain damage, confirming clinical obsevations that damage to
the selectively vulnerable cells is delayed (Petito, Feldmann, Pulsinelli & Plum, 1987).
The first report of delayed neuronal death following ischemia in animals was by Ito,
Spatz, Walker, & Klatzo (1975), who observed that in gerbils the number of damaged
cells increases progressively over a period of 24 to 72 hours after reperfusion.
Subsequent studies have confirmed this observation both in gerbils (Kirino, 1982) and
rats (Pulsinelli & Brierley 1979, Petito & Pulsinelli, 1984). Most striking is the finding
that CA1 hippocampal neurons do not show microscopic evidence of damage until 24
hours after reperfusion, and that the number of necrotic neurons increases until 72
hours after reperfusion. More recent studies have shown that although the primary
damage observed in CA1 occurs within a few days of the ischemic episode, there is
progressive degeneration of CA1 afferents over survival periods of 6-12 months after
ischemia (Mudrick & Baimbridge, 1989; Onodera, Aoki, Yae, & Kogure, 1990).

These features of ischemic brain damage are relevant both when considering the
mechanisms of damage (discussed in a later section), and also when developing animal
models of ischemia-induced amnesia. First, it is important to wait at least several days,
if not weeks or months, before testing animals on behavioural tasks, to allow as much
of the ischemia-induced damage to occur as possible. Second, it is important to assess
the extent and location of ischemia-induced cell loss in each experiment before
ascribing particular behavioural or memory deficits to particular patterns of cell loss;
the extent of damage is determined largely by the severity of the insult which may vary
between animals and procedures used.

**Monkeys models of ischemia-induced amnesia**

Two groups have modelled ischemia-induced amnesia in primates using,
respectively, permanent bilateral posterior cerebral artery occlusion (Bachevalier &
Mishkin, 1989) and 15 min bilateral carotid occlusion combined with hypotension
(Zola-Morgan, Squire, Rempel, Clower & Amaral, 1992). The bilateral posterior
cerebral artery occlusion model produces focal ischemia, but the pattern of cell loss
was similar to that reported following transient forebrain ischemia; the predominant
lesion was of CA1 and CA2 regions of the hippocampus, and the parahippocampal
gyrus (Bachevalier and Mishkin, 1989). The bilateral carotid occlusion model used by
Zola-Morgan et al. (1992) resulted in significant loss of pyramidal cells in CA1 and
CA2, and of somatostatin-containing cells in the hilus of the dentate gyrus, in the
absence of significant damage to CA3 or extrahippocampal structures.

In each of these studies ischemic monkeys were tested on a nonspatial object
recognition task; nonrecurring-items delayed nonmatching-to-sample (DNMS) (Mishkin
& Delacour, 1975). On each trial of DNMS a monkey is presented an unfamiliar
sample object which it has to displace to receive a food reward. Following a retention
delay, during which the sample object is hidden from view, the sample object is
presented again, along with a novel object. The monkey is rewarded for displacing the
novel object. DNMS is similar to recognition memory tests used in neuropsychological assessment; in order to perform DNMS the monkey must be able to distinguish between an object that was presented previously and one that was not. Similarly, in typical human recognition memory tests, a subject is presented with a list of items (e.g. words, pictures or nonsense syllables), and must identify which of the items on the list appeared on a different list that was presented previously (e.g. Squire & Shimamura, 1986). As discussed above, such recognition tasks are thought to assess explicit or declarative memory, as they require conscious recollection of specific information. It cannot be ascertained whether the DNMS performance of rats also requires declarative/explicit memory, as conscious recollection can only be inferred for animals. However, it is likely that DNMS performance by monkeys requires the same memory systems that underly declarative or explicit memory in humans. Recently it has been shown that patients with ischemia-induced amnesia are impaired relative to control subjects on the same DNMS task used to test monkeys (Squire, Zola-Morgan & Chen 1988). This clinical observation provides concrete evidence that, at least in humans, DNMS requires the structures that are damaged in ischemia, and is therefore a valid and sensitive test of recognition memory. It is therefore possible to compare directly the performance of humans and monkeys using the results obtained on DNMS.

Transient cerebral ischemia resulted in permanent deficits in performance on DNMS both in naive monkeys (Zola-Morgan et al 1992) and in monkeys that had received extensive DNMS training prior to ischemia (Bachevalier and Mishkin 1989). Monkeys with no training on DNMS prior to ischemia were able to learn the task with a delay of 8 s, but were impaired compared to controls at delays of 15, 60 and 600 s (Zola-Morgan et al 1992). When these same monkeys were retested several months
later, they took more trials than control monkeys to relearn the task to criterion, and having relearned the task, were again impaired at delays of 15, 60 and 600 s. Monkeys that had received extensive preoperative training on DNMS required more trials to reach the learning criterion after surgery than control monkeys, and were also impaired at delays of 10, 30, 60 and 120 s following ischemia (Bachevalier & Mishkin, 1989).

The performance of monkeys following ischemia is similar to that of amnesic patients; when tested on DNMS amnesic patients were able to learn the task with a delay of 5 s between presentation of the sample and the choice, although they required more trials to reach the learning criterion (90% correct on two consecutive days) than control subjects. When the delay between sample and choice was varied between 5, 15 and 60 s, the ischemic patients were severely impaired at all delays. After several hundred test trials amnesic patients performed as well as controls at the 5-s delay, but remained impaired on the 15-s and 60-s delays (Squire et al., 1988). These data indicate that impaired acquisition and poor performance on DNMS across various delays following ischemia are common across monkeys and humans. R.B. and other amnesic patients also show increased sensitivity to the imposition of delays on several other recognition tasks (Squire and Shimamura, 1986). While this delay dependent deficit was observed with the monkeys that did not receive preoperative training (Zola-Morgan et al., 1992), the monkeys that received training on DNMS prior to ischemia were impaired to the same extent across all delays (Bachevalier and Mishkin, 1989). There are therefore some subtle differences between the performance of monkeys and humans on recognition tasks.

Zola-Morgan et al (1992) also examined the performance of ischemic and control monkeys on two tasks that are sensitive to human amnesia, and two which are
analogous to tasks on which amnesic patients perform as well as control subjects. The ischemic monkeys showed no impairment on a skill learning task, and a pattern discrimination task, both of which are thought to rely on non-declarative memory. Surprisingly, they showed little or no impairment on the two tasks that have been shown to be sensitive to amnesia in humans; delayed object discrimination and concurrent discrimination.

Given the complementary behavioural findings in monkeys and humans, the monkey models can provide important information concerning the neuropathological changes that underly ischemia-induced memory impairments. The neuropathological profile of the ischemic monkeys in these experiments demonstrated the same pattern of selective vulnerability seen in humans. Moreover, the extent of damage was similar to that reported for the patient R.B., and the degree of impairment monkeys showed on DNMS was related to the extent of the hippocampal lesion. Taken together, these data are compatible with the hypothesis based originally on the clinical findings (Zola-Morgan et al., 1986) that selective damage to the hippocampal formation results in recognition memory deficits. It is therefore possible that prevention of the hippocampal cell loss resulting from ischemia would also prevent ischemia-induced amnesia.

**Rodent models of ischemia-induced amnesia**

brain damage were carried out by Bruce Volpe, Haker Davis and their colleagues, who assessed the performance of rats on two categories of spatial memory tasks: working memory tasks and reference memory tasks. Working memory tasks are tasks in which the relationship between stimuli, responses, and rewards varies from trial to trial. In contrast, reference memory tasks are tasks in which these relationships are constant across trials (Honig, 1978). This distinction between working memory tasks and reference memory tasks leads to an operational definition of working memory and reference memory as the processes that are used by an animal during performance of a working memory tasks or a reference memory task, respectively (Olton, Becker, & Handelmann, 1979). It has been suggested that different neural circuits or systems mediate these kinds of memory (Olton et al., 1979). The working memory-reference memory distinction is somewhat different from either the declarative-procedural or explicit-implicit distinctions discussed above, as both working memory and reference memory may involve information that is explicitly known and available for recall. For example, the knowledge that movies are shown at cinemas (reference memory), and the knowledge that Superman is on at the Hollywood this week (working memory) are both explicit and declarative memories. Furthermore, amnesic patients have as much difficulty learning new reference information as they do learning new working information (Squire, 1987). It has therefore been argued that working memory and reference memory are both sub-divisions of declarative memory, and that both may be impaired in human amnesia (Squire, 1987).

Despite the apparent confusion over the relevance of the working memory-reference memory distinction to human memory systems, and human amnesia (Squire, 1987), this distinction has frequently been applied to describe the effects of
hippocampal lesions in rats (e.g. Olton et al., 1979); in general, hippocampal lesions in rats have been shown to disrupt performance on working memory tasks, but not to affect performance of reference memory tasks. While rats with hippocampal lesions show deficits on some reference memory tasks (Morris, Garrud, Rawlins & O'Keefe, 1982), it is clear that a subset of working memory tasks are sensitive to hippocampal lesions, and that a subset of reference memory tasks are not. It is tasks that fall into these two categories have been used by Volpe and his colleagues to investigate the effects of ischemic brain damage on learning and memory in rats.

Using a radial arm maze, Volpe and colleagues measured the performance of rats on working memory and reference memory aspects of the same task simultaneously. This was done by baiting the same subset of arms on an 8-arm (5 arms baited) or 12-arm (7 arms baited) radial maze on all trials. Successful performance (entering baited arms only) requires the rat to learn and remember which arms of the maze are never baited, and should therefore be avoided (the reference memory component), and also requires the rat to remember that an arm has been entered and the food taken from that arm on a given trial, so that it can avoid re-entering the arm on the same trial (the working memory component).

Rats that received no training prior to ischemia were significantly impaired compared to control rats on both the working memory and reference memory aspects of the 8-arm maze task. Ischemic rats made significantly more working memory errors (re-entry of a previously chosen arm) and significantly more reference memory errors (initial entry of an arm that was never baited) than control rats (Volpe, Pulsinelli, Tribuna and Davis, 1984). After 75 trials there was no significant difference between the number of reference memory errors made by ischemic and control rats, although
ischemic rats were still impaired on the working memory aspect of the task (Davis, Tribuna, Pulsinelli and Volpe, 1986). In a separate experiment, reference memory performance was not impaired following ischemia in rats that were given extensive training on the 8-arm maze task prior to ischemia. In this experiment, the working memory performance of ischemic rats was impaired only over the first 10 trials of postoperative testing (Davis, Baranowski, Pulsinelli & Volpe, 1986). When the working memory task was made more difficult, by using a 12-arm radial maze with 7 arms baited, the working memory deficit was more substantial (Volpe, Pulsinelli, Simolke and Davis 1986). If rats were given more preoperative training (80 trial as opposed to 36 trials) on the same task, working memory recovered more quickly (Volpe, Davis & Colombo, 1989).

Taken together, these data indicate that initial acquisition of the invariant spatial information required for reference performance is impaired by damage to the brain structures affected in ischemia; although ischemic rats are able to acquire reference information, they require more trials to do so than controls. These structures or systems are not required once the reference aspect of the task has been learned. In contrast, the structures damaged in ischemia appear to be necessary for the acquisition of the information that varies across trials, and that is necessary for working memory performance. However, the working memory performance recovers following ischemia in rats that have preoperative training. The severity and duration of the working memory deficit is dependent on the complexity of the task, and the amount of preoperative training.

These findings prompted further behavioural analyses to examine whether the dissociation between reference memory and working memory performance on spatial
radial arm maze tasks might reflect a general ability of rats with ischemic brain damage to retain trial invariant information, despite impairments in learning new information on each trial. This was investigated by examining the performance of rats on an alternation task in a split stem T-maze (Volpe, Waczec & Davis, 1988). In this task rats had to learn which side of the stem to enter (the same on all trials i.e. trial-independent reference aspect), and which goal arm to enter (alternated each trial i.e. trial-dependent working aspect) in order to find food. Following ischemia, pretrained rats were unimpaired on stem choice performance, but were significantly impaired on goal arm choice compared to control rats. These results are consistent with a dissociation between reference memory and working memory deficits in rats with ischemic brain damage.

In each of the studies described above, transient forbrain ischemia was induced for 30 min using a modified version the 4VO method developed by Pulsinelli & Brierley (1979). This procedure resulted consistently in extensive bilateral loss of CA1 pyramidal neurons and less severe damage to CA2 and CA3 pyramidal neurons and the dorsolateral caudate nuclei. In some animals there was additional damage to thalamic and cortical cells (Davis & Volpe, 1990).

Two issues arise from these data. First, rats with ischemic brain damage demonstrate a memory impairment for new and variable spatial information, at least on the spatial tasks used in these experiments. This impairment is analogous to some of the memory impairments found in humans and monkeys following ischemia. For example, DNMS requires the ability to learn and use new and variable (nonspatial) information, and could therefore be classified as a working memory task. Therefore, while it is difficult to compare the performance of rats on spatial reference memory and working
memory tasks with the performance of humans and monkeys on nonspatial tasks designed to assess declarative or explicit memory, the results may yield similar conclusions. Also, behavioural characterisation of ischemic rats is useful regardless of the tasks used, as behaviour provides an important measure of the efficacy of treatments designed to prevent ischemic brain damage.

A second issue arising from these findings concerns the pattern of neuronal loss that underlies the ischemia-induced spatial working memory deficits in rats. Given that the ischemia-induced damage is not restricted to the hippocampus, it is possible that the extrahippocampal damage may contribute to the memory deficits. Control experiments have shown that rats with radiofrequency lesions of the dorsal striatum are not impaired on the working memory aspect of the split-stem T-maze task (Colombo, Davis & Volpe, 1989). In addition, large ibotenic acid lesions of the hippocampus, which produce extensive loss of cells in all fields including the CA1, result in a working memory deficit on the T-maze equivalent to that of ischemic rats, whereas lower doses of ibotenic acid, which produce less CA1 cell loss than ischemia, but approximately the same amount of total hippocampal damage as ischemia, result in no working memory deficit (Davis & Volpe, 1990). On the basis of these findings it was suggested that the ischemia-induced striatal cell loss did not contribute to the memory impairment, and that it could be accounted for by the CA1 cell loss. However, these data do not address the possibility that either combined damage of striatum and hippocampus, or other extrahippocampal damage produced by ischemia and high doses of ibotenic acid, may have produced the memory impairment. Therefore, the neuropathological basis of ischemia-induced spatial memory deficits cannot be confirmed on the basis of the present data.
Since the initial studies of Davis and his colleagues described above, several other groups have examined learning and memory in rats with ischemic brain damage. While the specific details of the tasks used in these studies have varied, most are similar to the radial maze and T-maze tasks described above along two dimensions; first, they each require rats to learn relationships between spatial stimuli, responses and rewards, and second, the relationships to be learned are either invariant (and can thus be discussed in the context of reference memory tasks), or vary across trials (and can be discussed in the context of working memory tasks). The general findings of these studies support the conclusions of Davis & Volpe (1990) that ischemic brain damage disrupts performance of working memory tasks, retards the initial acquisition of reference memory aspects of tasks, and has little or no impact on reference information learned prior to ischemia. However, some of the studies incorporate unique features (both in terms of behavioural characterisation of ischemic rats, and in terms of the neuropathological profiles) which add to our understanding of ischemia-induced memory deficits in rats. I will discuss these briefly.

Grotta, Pettigrew, Rosenbaum, Reid, Rhoades, & McCandless (1988), and Kiyota et al. (1991), using 30 min 4VO and both 5 and 20 min 4VO respectively, have replicated the original 8 arm radial maze experiment in which 5 of the 8 arms are baited (Volpe et al., 1984), with similar behavioural results. Ischemia resulted in a persistent working memory deficit in all rats, but little (Kiyota et al., 1991) or no (Grotta et al., 1988) reference memory deficit in rats with no preoperative training. Histological verification of the damage in the second of these studies revealed extensive CA1 cell loss in both the 5 min group and the 20 min group (Kiyota et al., 1991), and a significant correlation between CA1 damage and working memory performance. The
histological data supports the theory that the memory impairments were a result of CA1 cell loss, as it is unlikely that such a short period of ischemia would have resulted in much, if any, extrahippocampal cell loss. However, cells were not quantified in any other hippocampal cell fields, or outside the hippocampus, so this conclusion can only be tentative. Histological verification of neuronal damage was not conducted in the other experiment (Grotta et al., 1988).

Two studies have examined the performance of ischemic rats on a paired run alternation task in a T-maze, similar to the working memory aspect of the T-maze task described by Volpe, Waczec & Davis (1988). On this version of the T-maze task, there are two components to each trial. On the first part of a trial, the rat is forced to enter one of the goal areas, where it is rewarded. On the second part of the trial, the rat is allowed to chose between the two goal areas. It is rewarded only if it enters the goal area not entered during the first part of the trial. Ischemia induced by 30 min 4VO results in severe impairments on this working memory task in rats that are trained prior to ischemia (Ordy et al., 1988). 15 min 4VO results in similarly severe deficits in rats that have no preoperative training (Hagan & Beauchard, 1990). In both studies, after rats had reached asymptotic performance on the basic task, delays were interposed between the first and second parts of each trial. Although ischemic rats were less accurate at longer delays than at short delays, they were not more sensitive to the imposition of delays than were control rats. i.e. ischemic rats performed worse than control rats at all delays. Histological verification of the damage in CA1 was conducted in both studies; following 30 min 4VO there was extensive CA1 cell loss, and the working memory impairment was correlated with the degree of CA1 cell loss (Ordy et al., 1988). 15 min 4VO also resulted in extensive CA1 cell loss (Hagan & Beauchard,
1990). In this study cells were also quantified in other hippocampal cell fields and extrahippocampal structures, revealing moderate loss of cells in CA2, CA3 and dorsolateral striatum. The histological data reported in this study verify that 4VO for only 15 min results in significant extrahippocampal cell loss, leaving open the possibility that in all of the experiments utilising 4VO for 15 min or longer, the memory deficits may be a consequence of extrahippocampal damage. However, in terms of characterizing the memory deficits that result from ischemic brain damage these studies are extremely useful. First, the delayed alternation task is conceptually similar to the DNMS task used with monkeys (and humans), except that in this task the stimuli are spatial locations, whereas in the DNMS task the stimuli are objects. Also, on the T-maze, the same two spatial locations are used as stimuli on each trial, whereas on DNMS new objects serve as stimuli on each trial. However, the tasks are similar along another dimension; both incorporate specific delays between the training part of a trial and the test. Interestingly, rats were impaired at all delays, similar to the performance of pretrained monkeys on DNMS, and in contrast to naive monkeys and patients who are extremely sensitive to delays.

The final set of experiments have examined place learning in a water maze by rats with ischemic brain damage (Auer et al., 1989; Hagan & Beaughard, 1990; Jaspers et al., 1990; Kiyota et al., 1991; Nunn, Peillet, Netto, Sowinski, Hodges, Meldrum & Gray, 1991). On the basic (reference memory) task rats are required to learn the location of a submerged platform on the basis of spatial cues. The location of the platform remains constant across trials. Following ischemia induced by 2VO (Auer et al., 1989; Jaspers et al., 1990) or 4VO (Kiyota et al., 1991; Hagan & Beaughard, 1990; Jaspers et al., 1990; Nunn et al., 1991) rats were eventually able to learn the
location of the platform, although in some cases they required more trials to do so than controls (Jaspers et al., 1990; Hagan & Beaughard, 1990, Nunn et al., 1991). These findings suggest a slight impairment in reference memory performance. On a slightly different version—the place learning set task—the location of the platform remains constant across trials within a session, but changes between sessions. Auer et al. (1989) found that on the first trial of each session control rats tended to search for the platform in the old location, whereas ischemic rats showed no evidence of remembering this location. On subsequent trials, control rats learned the new location of the platform more quickly than ischemic rats. Although the information to be acquired within a session is invariant, there is a large working memory component to this task, as the relationships between spatial stimuli and rewards change across sessions. Therefore, although this task is not described easily by the reference memory - working memory distinction, the performance of ischemic rats is compatible with such a dissociation. The finding that ischemic rats do not tend to search for the platform in the old location on the first trial of each session is consistent with the idea that ischemia retards the acquisition of reference information. The rats with ischemia induced by 4VO showed a similar pattern of cell loss as has been described in other experiments; extensive CA1 cell loss and, in those experiments in which other areas were examined, moderate cell loss in CA2, CA3 and dorsolateral striatum. The pattern of cell loss following 2VO (without hypotension) for 24 min (Jaspers et al., 1990) or for 9 min combined with systemic hypotension (Auer et al., 1989), was more restricted. Following bilateral carotid occlusion with no hypotension there was no evidence of neural destruction in the hippocampal formation, or in any extrahippocampal structures (Jaspers et al., 1990). These data are intriguing, given the significant (albeit small) reference memory
impairment. One explanation is that the transient reduction in cerebral blood flow may have induced longlasting functional impairments that was not manifested as cell loss. 2VO combined with hypotension for 9 min resulted in neuronal necrosis limited to 50% of the CA1 field of the hippocampus, and some loss of dentate hilar neurons (Auer et al., 1989). This experiment is significant in that it demonstrates an impairment on the learning set water maze task in rats with ischemic damage that appears to be limited to the hippocampal formation. However, as with the other spatial tasks discussed in this section, the behavioural results are hard to interpret with reference to the results of experiments on humans.

In summary, the general finding is that ischemia induced either by 4-vessel occlusion, or by 2VO (with or without hypotension) results in deficits on spatial working memory tasks, and to a lesser degree on spatial reference memory tasks. The incorporation of delays in a spatial working memory task does not significantly affect the impairment--rats are impaired equally at short and long delays. The predominant lesion in these rat models of ischemia involves pyramidal neurons in the CA1 field of the hippocampal formation, although in most cases extrahippocampal damage is also evident or can be inferred. Exceptions to this are deficits on a place learning set task on the water maze in rats with a selective lesion of CA1 and hilar neurons, and a mild reference memory impairment in rats with no apparent damage following incomplete ischemia induced by 24 min 2VO with no hypotension. The results of most of these studies are consistent with the hypothesis that ischemia-induced hippocampal damage is responsible for memory deficits following ischemia, although in few cases is this verified. The relation of these findings to ischemia-induced memory deficits in humans is difficult to assess given the need to compare performance on such widely disparate
tasks. The use of non-spatial tasks for rats that are analogous to those used to assess humans and monkeys would enable comparisons between the species, and may also provide a more appropriate rat model for assessing the effects of ischemic brain damage.

**MECHANISMS OF ISCHEMIC BRAIN DAMAGE**

All tissues are dependent on an adequate blood supply to provide oxygen and substrates for energy production. The mammalian brain is particularly dependent on a constant supply of blood as it has a high metabolic rate, but low storage capacity for oxygen and small reserves of high energy phosphates and carbohydrates (Rehncrona, 1986). Consequently, interruption of cerebral blood flow leads rapidly to a disruption of brain energy metabolism (Kaplan, Dimlich, Biros & Hedges, 1987). It is generally assumed that the precipitous decrease in adenosine triphosphate (ATP) is the triggering event in ischemic brain damage (Seisjo, 1988). The following sections will provide a description of the events triggered by this initial loss of energy, and mechanisms that have been proposed to account for ischemic brain damage, and the pattern of selective cell loss seen in global ischemia.

**Events triggered by disruption of brain energy metabolism**

Approximately 50% of the energy metabolism of neurons is used in the transport of ions (Siesjo, 1978, 1988). Transport of ions across the cell membrane is essential for normal ion homeostasis. In healthy neurons, membrane conductances allow influx of sodium, calcium and H⁺ and efflux of potassium. To maintain the ionic gradients which underlie the normal resting potential of neurons, ATP-dependent
mechanisms counteract these membrane conductances: The ATP-dependent sodium-potassium pump transports sodium out, and potassium into neurons, while calcium is extruded from the intracellular compartment by ATP-dependent translocation across the membrane. Calcium homeostasis is also maintained by two additional ATP-dependent mechanisms: transport of calcium out of the cell across the plasma membrane by sodium-calcium exchange, the energy for which is derived from the transmembrane sodium gradient, which is created in turn by the sodium-potassium ATP-ase, and ATP-dependent sequestration of calcium by the smooth endoplasmic reticulum and synaptic vesicles.

Disruption of cellular energy metabolism during ischemia results in the arrest of these ATP-dependent processes and dissipation of the ionic gradients, resulting in net efflux of potassium and influx of sodium, calcium and chloride (Hansen, 1985).

Consequences of the disruption of ion homeostasis - calcium as a mediator of ischemic brain damage

The disruption of ion homeostasis that results from ATP depletion during ischemia leads to the accumulation of intracellular free calcium (Raichle, 1983; Siesjo & Weiloch, 1985, Greenberg, Uematsu, Araki, & Reivich, 1991) as a result both of calcium influx, and of calcium release from endoplasmic reticulum and mitochondria. Calcium influx can occur both via calcium channels (both voltage-operated and ligand gated), and via the sodium-calcium antiporter, which works in reverse when sodium levels inside the cell are high. The massive increase in intracellular free calcium is thought to be a major initiator of irreversible ischemic neuronal damage (Siesjo, 1981, Farber et al 1981, Raichle, 1983).
While the specific biochemical and metabolic mechanisms by which elevated calcium levels cause cell death remain to be elucidated, it is clear that intracellular calcium can activate many events that might contribute to cellular dysfunction. For example, during ischemia free calcium activates membrane phospholipases, which break down membrane phospholipids, and thereby cause structural membrane alterations (Siesjo, 1990). Phospholipids that are broken down are usually recycled through a series of energy-dependent reactions. However, during ischemia these reactions cannot occur, such that these vital phospholipids become depleted (Farber 1981). Degradation of phospholipids leads to the accumulations of polyunsaturated free fatty acids, including arachidonic acid. These polyenoic fatty acids may perturb membrane structure and function (Siesjo, 1990), and arachidonic acid is a potent inducer of cellular cytotoxic edema (Chan, Fishman, Longar, Chen, & Yu, 1985). In addition, during reoxygenation (which occurs during reperfusion), arachidonic acid is metabolised resulting in the formation of prostaglandins and leukotrienes. These ecosanoid species are capable of affecting cell function in a detrimental fashion (Raichle, 1983). Potentially more dangerous to cell integrity is the production of free radical species as a by-product of this breakdown of arachidonic acid (Schmidley, 1990). Free radicals are species that have an unpaired electron in their outermost orbital. Covalent chemical bonds usually consist of a pair of electrons sharing an orbital. Consequently, free radicals are extremely reactive, and are capable of peroxidising membrane proteins and lipids, thus degrading membrane integrity. Free radical production may also be triggered directly by high intracellular calcium, as calcium can initiate the conversion of xanthine dehydrogenase to xanthine oxidase which is a rich source of superoxide radicals (Dykens, Stern & Tracker, 1987).
Substantial evidence has accumulated supporting a role for free radicals in ischemic cell damage (Demopoulos 1977, Chan & Fisherman, 1985, Chan et al., 1985; Siesjo & Weiloch, 1985), and they may be responsible for the ability of arachidonic acid to induce cytotoxic and vasogenic edema (Chan, Fishman, Caronna, Schmidley, Prioleau, & Lee, 1983).

Sustained high levels of intracellular free calcium can also have devastating effects on proteins (Siesjo, 1990). One example is the progressive proteolysis of neurofilaments and other vital components of the neuronal cytoskeleton by calcium-activated proteases (calpains) (Schlaepfer & Zimmerman, 1985; Arai, Vanderklish, Kessler, Lee, & Lynch, 1991). Calcium can also affect cytoskeletal proteins by non-proteolytic mechanisms. For example, the tau factor that is necessary for assembly of microtubuli can be bound in a complex with calcium and calmodulin. If this occurs, microtubuli will disassemble into tubulin subunits (Seisjo & Wieloch 1985).

Intracellular calcium can also activate mechanisms that result in the phosphorylation of both pre and post-synaptic proteins causing long term changes in membrane function and increased susceptibility to subsequent neuronal excitation and calcium accumulation. For example, phosphorylation of pyruvate dehydrogenase reduces the ability of mitochondria to sequester calcium (Browning, Baudry, Bennet, & Lynch, 1981). If this occurs during ischemia, cells would be less capable of maintaining calcium homeostasis in the period following reperfusion, and would therefore be more susceptible to calcium influxes during the postischemic period than "normal" cells. Similarly, phosphorylation of receptor proteins on postsynaptic membranes may alter their affinity for ligands, or their channel properties, making cells more excitable. Finally, phosphorylation of presynaptic membrane proteins may
enhance transmitter release to a given stimulation. Recent evidence suggests that these kinds of mechanisms may be able to explain the maturation phenomenon or delayed neuronal death that characterises ischemic damage. For example, calcium influx evoked by electrical stimulation is enhanced 6 hours following transient forebrain ischemia, and this period of increased influx precedes delayed neuronal degeneration (Andine, Jacobson & Hagberg, 1988). It has been shown independently that neuronal degeneration is preceded by enhanced excitatory transmission in the postischemic period (Urban, Neill, Crain, Nadler & Somjen, 1989, 1990). The mechanism of delayed neuronal death envisaged here is that ischemia causes massive calcium influx which, through activation of enzymes that phosphorylate proteins, sensitizes neurons such that normal synaptic activity during the postischemic period will result in damage (Diemer, Johanson & Jorgensen, 1990). This process probably contributes to ischemic cell loss in combination with the other processes described above.

While there is substantial experimental evidence supporting the roles of calcium and free radicals in ischemic cell damage, the events described so far cannot account for the selective vulnerability of particular neurons to global ischemia; all cells lose their calcium homeostasis. To explain this vulnerability, it is necessary to postulate additional factors or mechanisms that render some neurons more sensitive than others to a disruption in calcium homeostasis. Possible contributing factors are that vulnerable neurons have a higher density of ligand-gated and/or voltage-gated calcium channels in their membranes, that they have fewer intracellular calcium binding proteins, or that they have a preponderence of intracellular and membrane proteins that are have high calcium-sensitivity.
The most widely-accepted theory that accounts, to a large extent, for the pattern of brain damage caused by ischemia is the excitotoxic theory (Rothman & Olney, 1986, Choi, 1988, Albers, Goldberg, & Choi, 1989).

The excitotoxic theory of ischemic brain damage

The excitotoxic theory of ischemic brain damage (Rothman & Olney, 1986) postulates that a key event mediating the accumulation of intracellular calcium in ischemia is calcium influx and calcium release from intracellular stores, both stimulated by the activation of glutamate receptors. It is well established that, at appropriate concentrations, glutamate can be cytotoxic to most central neurons (Olney & Sharp, 1969; Olney, Ho, & Rhee, 1971). Insight into the mechanisms of glutamate-induced cytotoxicity, and similarities between the pathological changes observed following glutamate-mediated cell death and ischemia have led to the theory that the excitotoxicity of glutamate may play a major role in cerebral ischemia.

Five main subgroups of excitatory amino acid receptors are currently recognised on the basis of distinct pharmacological and molecular biological profiles (Watkins, Krogsgaard-Larsen, & Honore, 1990; Krogsgaard-Larsen, 1992), and at least four of these receptor subtypes (the N-methyl-D-aspartate (NMDA), kainate (KA), alpha-amino-3-hydroxy-5-methyl-4-isoxaxolepropionic acid (AMPA), and metabotropic receptors) may be involved in glutamate-mediated cytotoxicity. The NMDA, KA and AMPA receptors are linked to ion channels, with distinct properties: KA opens a small conductance channel permeable to sodium and potassium, AMPA opens an intermediate channel permeable to sodium and potassium, and NMDA opens a large conductance channel which is permeable to calcium as well as to sodium and potassium.
A unique feature of the ion channel associated with the NMDA receptor is that it is usually blocked by magnesium in a voltage-dependent fashion (Mayer, Westbrook, & Guthrie, 1984; Nowak, Bregestovski, Ascher, Herbet, & Prochiantz, 1984). It has recently been demonstrated that glycine can potentiate the conductance mediated by NMDA receptor activation (Johnson & Ascher, 1987) and it has been suggested that glycine may be an essential co-agonist at the NMDA receptor-channel complex. Consequently, it is now thought that simultaneous binding of agonists to the NMDA and strychnine-insensitive glycine binding sites is required, with concomitant cell depolarization to relieve the magnesium block, for ion conductance through the channel (Dingledine, Kleckner & McBain, 1990; Kleckner & Dingledine, 1988; Lehmann, Colpaert & Canton, 1991; Lerma, Zukin & Bennet, 1990). In addition to these ionotropic receptors there is a metabotropic glutamate receptor that is coupled to inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol turnover (Sladeczeik, Pin, Recasens, Blockaert, & Weiss, 1985; Schoepp, Blockaert, & Sladeczek, 1990). Each of these 4 types of excitatory amino acid receptors may contribute to glutamate mediated toxicity, as glutamate is a mixed agonist, acting on each of these receptor subtypes.

Two types of damage can occur as a result of glutamate receptor activation: osmolytic damage and calcium-related damage (Rothman and Olney 1986). Osmolytic damage may be triggered by activation of the non-NMDA ionotropic receptor subtypes, allowing influx of sodium down its electrochemical gradient. Chloride, moving passively down its electrochemical gradient, follows sodium. In order to maintain osmotic balance, water diffuses into the cell, resulting in cell swelling and lysis.
Activation of glutamate receptors can also result in accumulation of intracellular free calcium, by any of several mechanisms. As discussed above, accumulation of calcium can be pathological, particularly in circumstances of reduced or arrested ATP production. The most prominent mechanism of glutamate-mediated calcium accumulation is thought to involve calcium influx, primarily via the NMDA receptor-linked channel (Choi, 1985, 1987, 1988a, Rothman, Thurston, & Hauhart, 1987). The scenario here is that the non-NMDA and NMDA receptors act in concert to trigger calcium influx: activation of the non-NMDA ion-linked receptors by glutamate causes influx of sodium, and subsequent cell depolarisation. This cell depolarisation, in combination with glutamate and glycine binding to their respective sites on the NMDA receptor-channel complex, results in NMDA channel opening, allowing calcium influx. There are two additional ways in which glutamate receptor activation may contribute to intracellular calcium accumulation: first, sustained membrane depolarisation due to activation of the NMDA and/or non-NMDA cationic conductances will activate voltage-operated calcium channels (VOCCs) allowing additional calcium influx (Choi, 1988b). Second, activation of the metabotropic glutamate receptor stimulates IP3 and DAG which, in turn, can promote the release of calcium from intracellular stores, including the smooth endoplasmic reticulum (Taylor, 1987). In addition, stimulation of polyphosphoinositol hydrolysis by activation of metabotropic receptors at the presynaptic level (which have been demonstrated by autoradiography and in situ hybridisation (Nicoletti et al., 1992), may result in a prolongation of glutamate release, thus prolonging the process.
Evidence in support of the excitotoxic theory of ischemic brain damage

There are several lines of evidence, both in vivo and in vitro, that support the theory that ischemic neuronal damage is mediated, at least in part, by calcium accumulation triggered by the activation of glutamate receptors. The strongest in vivo evidence rests on the finding that glutamate receptor antagonists, in particular those capable of blocking the NMDA receptor, reduce the neuronal damage induced by both global ischemia (Gill, Foster, & Woodruff, 1987; Iversen, Woodruff, Kemp, Foster, Gill, & Wong, 1988; Rod & Auer, 1989; Simon, Swan, Griffiths, & Meldrum, 1984; Swan, Evans, & Meldrum, 1988; Swan & Meldrum, 1990) and focal ischemia (Duverger, 1987; George, Goldberg, Choi, & Steinberg, 1988; Germano, Pitts, Meldrum, Bartkowski, & Simon, 1987; Ozyurt, Graham, Woodruff, & McCulloch, 1988; Prince & Feeser, 1988). However, there are exceptions (e.g. Buchan, Li & Pulsinelli, 1991). These will be discussed in more detail below. Further support for a role for glutamate in ischemic brain damage comes from in vivo studies demonstrating that transection of glutamatergic afferents attenuates ischemia-induced loss of pyramidal CA1 neurons (Wieloch, Lindvall, Blomqvist, & Gage, 1985, Onodera, Sato, & Kogure, 1986). A third line of evidence is that both ischemia (Diemer & von Lubitz, 1983; Johansen, Jorgensen, von Lubitz, & Diemer, 1984) and glutamate exposure (Olney, 1978) result in dendrosomatic, axon sparing lesions. Postsynaptic glutamate receptors are assumed to reside in apical dendrites, and especially dendritic spines, so calcium influx via NMDA receptors would be confined to these regions. Finally, several studies utilising the in vivo microdialysis technique have demonstrated that extracellular concentrations of glutamate and aspartate increase dramatically during ischemia (Benveniste, Drejer, Schousboe, & Diemer, 1984; Drejer, Benveniste,
Corroborative evidence for the involvement of glutamate in ischemia can be inferred from *in vitro* studies: NMDA antagonists have been shown to be protective against anoxia and hypoxia in vitro (Clark & Rothman, 1987; Golberg, Viseskul, & Choi, 1988; Goldberg, Weiss, Pham, & Choi, 1987; Kass, Chambers, & Cottrell, 1989; Pohorecki, Becker, Reilly, & Landers, 1990; Rothman, 1984; Rothman, Thurston, Hauhar, Clark, & Solomon, 1987; Weiss, Goldberg & Choi, 1986); and cultured hippocampal neurons deprived of their synaptic input (which is presumed to be glutamatergic) survive hypoxia (Rothman, 1983).

There is also circumstantial evidence from *in vivo* and *in vitro* electrophysiological studies that one of the events that triggers the loss of ion homeostasis may be secondary to glutamate release. Close examination of the time course of the early events that occur during ischemia in experiments in which extracellular concentrations of ions are measured using ion-sensitive electrodes has revealed that the first event is a gradual increase in extracellular potassium, and this is followed by rapid decreases in the extracellular concentrations of calcium, sodium and chloride (Siesjo & Bengtsson, 1989, Hanson, 1985). This pattern of events corresponds with the observations from *in vitro* studies in which intracellular recordings are made from hippocampal pyramidal neurons during anoxia: the first consistent change after the onset of anoxia is a pronounced hyperpolarisation accompanied by a decrease in membrane resistance. This is followed by a depolarisation and further decrease in membrane resistance (Fujiwara, Higashi, Shimoji, & Yoshimura, 1987). One explanation for the early hyperpolarisation and increase in extracellular potassium is
that depletion of ATP may cause an increase in potassium conductance directly by causing opening of ATP-sensitive potassium channels (Fujiwara et al., 1987). These channels, which have been identified in the brain (Ashford, Sturgess, Trout, Gardner, & Hales, 1988, Mourre, Ben Ari, Bernardi, Fosset, & Lazdunski, 1989), are closed when intracellular ATP levels are normal but open when ATP levels are reduced, resulting in efflux of potassium from the cell. An alternative explanation is that the hyperpolarization and increase in extracellular potassium are the result of potassium conductance through calcium-dependent potassium channels. This conductance may be stimulated by an increase in intracellular calcium that occurs as a result of the arrest of ATP-dependent calcium sequestering processes. Recent evidence showing that modulators of the ATP-dependent potassium channels have no effect on the anoxia-induced hyperpolarization favour the second hypothesis (Ben Ari, 1990). Given that an increase in extracellular potassium precedes the period of depolarisation and the decrease in extracellular sodium and calcium, it is plausible that the depolarization could be triggered by high extracellular potassium. Specifically, high extracellular potassium would depolarize axon terminals, opening voltage-sensitive calcium channels which in turn would elicit calcium-dependent neurotransmitter release. This would account for the observation that in addition to glutamate, the extracellular concentrations of several other transmitters increase dramatically during ischemia (e.g. Globus, Busto, Dietrich, Martinez, Valdes & Ginsberg, 1988, Wood, Coury, Blaha & Phillips, in press, Damsma, Boisvert, Mudrick, Wenkstern, & Fibiger, 1990). The actions of glutamate on its post-synaptic receptors may then precipitate the rapid depolarisation that follows the gradual potassium efflux, by allowing influx of sodium and calcium as discussed above.
Potassium-stimulated calcium-dependent release of glutamate may not be the only mechanism by which extracellular levels of glutamate become elevated during ischemia. As discussed above, mobilization of calcium from intracellular stores may be stimulated by presynaptic metabotropic receptor activity, which would contribute to calcium-dependent release of glutamate. In addition, glutamate release could occur by a calcium-independent mechanism during ischemia (Sanchez-Prieto & Gonzalez 1988, Ikeda, Nakazawa, Abe, Kaneko, & Yamatsu, 1989). Of interest in this regard is a recent report of free-radical stimulated release of glutamate during ischemia (Pellegrini-Giampietro, Cherici, Alesiani, Carla, & Moroni, 1990). Finally, increases in extracellular glutamate during ischemia could be mediated, in part, by the inhibition or arrest of mechanisms responsible for glutamate uptake and/or inactivation. Glutamate uptake by glia is thought to be achieved by a carrier that transports three sodium ions into glia, and one potassium cell ion out (Barbour, Brew, & Atwell, 1988). The high extracellular levels of potassium during ischemia may therefore inhibit, or even reverse this process.

Given the large body of evidence reviewed above it seems likely that ischemic brain damage is mediated, at least in part, by the excitotoxic actions of glutamate. How, then, can the excitotoxic theory of ischemic brain damage account for the selective vulnerability of particular cells to global ischemia? Both the pattern of glutamate innervation and the distribution of glutamate receptors contribute to the selective vulnerability of particular neurons to ischemia. First, the distribution of vulnerable neurons corresponds well with the pattern of glutamatergic innervation (Cotman, Monaghan, Ottersen & Storm-Mathisen, 1987). Second, receptor autoradiography has shown that the distribution of glutamate receptors, particularly the
NMDA receptor subtype, corresponds extremely well with the pattern of selective vulnerability (Greenamyre, Olson, Penney, & Young, 1985). However, exceptions to these generalizations exist as dentate and CA1 both have dense glutamatergic innervation and a high density of NMDA receptors, yet CA1 neurons are more vulnerable. On the other extreme, cerebellar Purkinje cells are among the most vulnerable neurons yet have a low density of NMDA receptors. Recently it has been noted that the distribution of AMPA receptors also corresponds well with the distribution of vulnerable neurons (Diemer et al., 1990), and it has been suggested that Purkinje cell vulnerability in the cerebellum may be attributable to the high density of non-NMDA receptors. Molecular biological evidence suggests that there may be many non-NMDA receptor types, some of which allow calcium flux, depending on the assembly of receptor subunits, (Barnard et al., 1992; Boulter et al., 1992). This is corroborated by recent electrophysiological findings in cultured hippocampal neurons (Ozawa, Ino, Tsuzuki, & Takeuchi, 1992) indicating that some non-NMDA ionotrophic glutamate receptor channels may be permeable to calcium. It is therefore feasible that cerebellar vulnerability could still be mediated by calcium influx via non-NMDA glutamate receptors. However, there are almost certainly additional factors which contribute to the pattern of selective vulnerability in ischemia. These may include the density of voltage operated calcium channels, the ability of cells to buffer free intracellular calcium, and the abundance of proteins that are susceptible to calcium-activated phosphorylation.

Neurons have developed several mechanisms to buffer intracellular calcium levels within strict spatial and temporal constraints. The energy-dependent mechanisms for maintaining physiologically useful levels of calcium, such as sequestration by the
smooth endoplasmic reticulum and translocation across the cell membrane, are clearly compromised during ischemia. However, many neurons also possess fast calcium buffering capabilities, provided by intracellular calcium binding proteins such as parvalbumin, calbindin and calmodulin (Baimbridge, Celio & Rogers, 1992; Persechini, Moncrief & Kretsinger, 1989). The heterogeneous distribution of these proteins could contribute to the pattern of selective vulnerability; neurons that synthesise large amounts of calcium binding proteins would be expected to have greater calcium-buffering capability, and may therefore be more resistant to ischemia than neurons that do not. Consistent with this is the suggestion that the resistance of GABA-ergic CA1 interneurons in the gerbil and rat may be a function of their high levels of parvalbumin (Nitsch, Scotti, Sommacal, & Kalt, 1989; Mudrick & Baimbridge, 1989). In addition, administration of the calcium chelator BAPTA protects hilar neurons (which contain none of the known calcium-binding proteins, and which are vulnerable to ischemia), against the damage caused by prolonged stimulation of afferent pathways (Scharffman & Schwartzkroin, 1989). A recent study assessed the relationship between the neuronal content of calbindin and parvalbumin, and vulnerability to ischemia (Freund, Busaki, Leon, Baimbridge, & Somogyi, 1990). Within the hippocampus, calbindin-immunoreactivity is normally seen in interneurons throughout CA1 to CA3, in granule cells of the dentate gyrus and in pyramidal neurons in the superficial pyramidal cell layer of CA1 (Mudrick & Baimbridge, 1991). Hilar neurons and CA3 pyramidal neurons do not express calbindin. The calbindin-reactive interneurons and granule cells are resistant to ischemia, whereas the calbindin-negative hilar neurons are extremely vulnerable. However, calbindin-immunoreactivity does not predict resistance to ischemia in all cells, as the superficial calbindin-immunoreactive CA1 pyramidal
neurons are among the most vulnerable to ischemia. The distribution of parvalbumin within the hippocampus corresponds better to the pattern of neuronal sparing in ischemia; a subpopulation of nonpyramidal neurons in all cell fields shows parvalbumin reactivity, and these neurons appeared intact after ischemia, although there was partial loss of parvalbumin-positive non-pyramidal neurons in the CA1 field of some animals. There was a similar lack of correspondence between calcium-binding protein content and resistance to ischemia in other areas of the brain. It therefore seems unlikely that the presence of calcium-binding proteins is sufficient to protect against ischemia-induced cell death, although it is possible that calcium-binding proteins contribute to the pattern of vulnerability, in combination with the distribution of glutamate receptors. For example, the low density of NMDA receptors on CA3 pyramidal neurons may render them resistant to ischemia although they have no calbindin or parvalbumin immunoreactivity. Conversely, the high calbindin content of dentate granule cells may offer protection, despite the high density of NMDA receptors on granule cell dendrites.

The lack of a consistent relationship between the calcium-binding protein content of neurons and their vulnerability to ischemia is not altogether surprising; it is likely that those cells which have developed mechanisms to buffer intracellular calcium efficiently using these proteins are those in which calcium is employed for normal functions, such as the mediation of neuronal plasticity and activation of second messenger systems. Therefore, these cells may have developed efficient calcium buffering systems because they are more sensitive to changes in intracellular calcium concentration than cells in which calcium does not play such a specialised role.

In summary, compelling evidence has accumulated in support of the theory that ischemic brain damage is mediated, at least in part, by the action of glutamate at its
receptors. Elevated levels of glutamate during ischemia activate receptors, leading to an increase in intracellular calcium. This may occur via activation of any of KA, AMPA, NMDA and metabotropic receptors. A prominent hypothesis is that calcium influx via the NMDA receptor plays a major role in initiating calcium influx, but calcium influx via voltage sensitive calcium channels, secondary to activation of non-NMDA and/or NMDA receptors, may also contribute. Intracellular calcium can then activate a series of events, including (upon reoxygenation of the brain) free radical production and activation of kinases. It is not thought that this initial calcium accumulation is directly responsible for cell death (Diemer et al., 1990). Rather, the alterations in membrane function act to sensitize the cell to subsequent transmission so that normal transmission allows further calcium influx, and this is deadly. The excitotoxic theory of ischemic brain damage can account to a large extent both for the pattern of selective vulnerability and for the delayed neuronal death seen following transient forebrain ischemia. Consequently, many studies have examined the protective effects of drugs that interfere with glutamatergic activity in ischemia. These will be reviewed briefly in the following section.

**PHARMACOLOGICAL INTERVENTION IN CEREBRAL ISCHEMIA**

Several different strategies have been adopted with the aim of preventing ischemic brain damage by means of interrupting glutamatergic receptor activation. These strategies include blocking the NMDA receptor or the channel associated with the NMDA receptor, blocking non-NMDA glutamate receptors, and interfering with glutamate release. The protective properties of drugs have been assessed both in vivo, using models of focal and global ischemia, and in vitro. For the purposes of this review
I will discuss only the results from experiments in which the efficacy of pharmacological intervention has been assessed in models of global ischemia \textit{in vivo}.

**NMDA antagonists**

The development of a wide range of NMDA receptor antagonists over recent years has allowed evaluation of the therapeutic potential of pharmacological blockade of NMDA-receptor mediated calcium influx. There are several distinct sites within the NMDA receptor-channel complex at which drugs may act to attenuate glutamate's effects (see Figure 1). Competitive antagonists such as 2-aminophosphonopentanoate (AP5), 2-amino-7-phosphonoheptanoate (AP7), 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP) and \textit{DL}-\textit{cis}-4-phosphonomethyl-2-piperidine carboxylate (CGS 19755) directly block the site at which glutamate and NMDA bind. The \textit{in vivo} efficacy of some of these drugs (AP5 and AP7) is limited as they are extremely polar, and have negligible access to the brain following systemic administration. However, the more recently developed CPP and CGS 19755 can cross the blood-brain barrier more easily, and thereby have greater therapeutic potential. Glutamate's actions at the NMDA receptor-channel complex can also be blocked by noncompetitive antagonists. These drugs, which readily cross the blood-brain barrier, do not compete for binding at glutamate recognition sites, but rather bind to sites within the NMDA receptor-activated channel, thereby impeding ion flux. These non-competitive antagonists exhibit use-dependency; they can only gain access to the binding site within the channel if the channel is open, and therefore rely on agonist activation of the NMDA receptor. Similarly, \textit{recovery} from channel block by noncompetitive antagonists is dependent on NMDA agonist binding. There are several classes of non-competitive NMDA
Figure 1. Schematic diagram of the NMDA receptor-channel complex, illustrating the sites at which different classes of agonists and antagonists bind (Adapted from Young & Fagg, 1990).
NMDA receptor-channel complex
antagonists including the dissociative anesthetics ketamine and phencyclidine (PCP), the dextrorotatory morphians dextromethorphan (DM) and dextrophan (DX), the dibenzocycloheptamine MK801, and the sigma opiates, including SKF 10047. A third site of action for antagonists is the glycine binding site. As discussed above, glycine binding is thought to be required, in combination with agonist binding to the NMDA recognition site, for NMDA channel opening. Competitive antagonists at the glycine binding site including 7-chlorokynurenic acid and HA699 have been shown to inhibit NMDA-receptor mediated channel opening (Kemp et al., 1988; Kloog, Lamdani-Itkin, & Sokolovsky, 1990). The ability of this class of drugs to cross the blood-brain barrier has not yet been established, but is thought to be low, due to the polar nature of the compounds.

Several studies have examined the therapeutic efficacy of the competitive and noncompetitive NMDA antagonists against ischemic neuronal damage in a variety of animal models. Early studies indicated that intrahippocampal administration of the competitive antagonist AP7 prior to transient forebrain ischemia in rats significantly attenuated neuronal degeneration in the hippocampus (Simon et al., 1984, Swan et al., 1988). Similar protective effects have been reported following systemic administration of CGS 19755 up to 30 min following reperfusion after 4VO (Grotta et al., 1988). This finding has recently been extended to gerbils, in which systemic administration of AP7 (Boast, Gerhardt, & Janak, 1987), CGS 19755 or CPP (Boast, Gerhardt, Pastor, Lehmann, Etienne, & Liebman, 1988) significantly reduces hippocampal damage induced by bilateral carotid occlusion. In these experiments AP7 and CGS 19755, but not CPP, were effective if administered after the period of ischemia. However, not all studies report protective effects of competitive NMDA antagonists; for example AP7
did not protect against hippocampal damage in a rat 4VO model (Plum, Slivka, Block, & Pulsinelli, 1987).

Studies with noncompetitive NMDA antagonists have yielded even more mixed results. MK801 has been reported to attenuate cell loss in the hippocampus if administered prior to 5 min bilateral carotid occlusion in gerbils (Gill, Foster & Woodruff, 1987a), or bilateral carotid occlusion combined with systemic hypotension in the rat (Church, Zeman, & Lodge, 1988; Rod & Auer, 1988). In some cases MK801 has also offered protection if administered after reperfusion (Gill, Foster, & Woodruff, 1988; Rod & Auer, 1988). Recent electrophysiological evidence is also consistent with a protective effect of MK801 against the loss of CA1 pyramidal cell function induced by transient forebrain ischemia (Gill & Kemp, 1989). Extracellular recordings were made from hippocampal slices taken from gerbils four days after 5 min bilateral carotid occlusion. No postsynaptic field potential or population spike could be recorded from the CA1 region in these slices, although a presynaptic fibre volley was present, indicating that the afferent input to CA1 was still intact. In contrast, normal CA1 field potentials, population spikes and synaptic responses were obtained from hippocampal slices taken from gerbils that were treated with MK801 1 h prior to carotid occlusion. However, other studies have failed to demonstrate neuronal protection by MK801 administered before or after transient forebrain ischemia in rats (Buchan, Li & Pulsinelli, 1991; Nellgard, Gustafson, Hansen, Lauritzen, & Wieloch, 1989; Wieloch, & Nellgard, 1991). Furthermore, it has been suggested that some of the earlier reports of protection by MK801 may be attributable to its hypothermic effects (Buchan & Pulsinelli, 1990b; Diemer, Johansen & Jorgensen, 1990). This claim has been challenged by Gill & Woodruff (1990) who have demonstrated that MK801 is
efficacious when administered prior to ischemia even if the brain temperature of the gerbils (assessed by means of a thermistor placed close to the temporalis muscle) is maintained at 37° for 24 h after reperfusion. Similar mixed findings have been reported concerning the protective effects of another noncompetitive NMDA antagonist, ketamine, against ischemic damage in models of global ischemia in vivo (Church et al., 1988; Marcoux, Goodrich, & Dominick, 1988; Meldrum, Evans, Swan, & Simon, 1987).

Despite the mixed reports concerning the protective effects of NMDA antagonists, the positive findings are consistent with a role for calcium influx via the NMDA receptor during ischemia. In particular, MK801 and APH appear to have the most efficacy when administered prior to ischemia, indicating that NMDA receptor activation contributes to delayed neuronal death during the period of ischemia itself. This observation is consistent with the hypothesis that calcium influx via the NMDA receptor-linked ionophore during ischemia sensitises neurons to events that occur during the postischemic period (Diemer et al., 1990), and after this time, NMDA receptor activation plays only a minor role in the development of delayed neuronal death. One proposal for the lack of a protective effect of the non-competitive NMDA antagonists in some experiments is that under conditions in which the membrane is markedly depolarised (as is the case during ischemia), the ability of these antagonists to block the channel is attenuated (Miller, 1987).

Although NMDA receptor antagonists are potentially therapeutic if administered prior to ischemia, there are several negative effects that might prohibit their clinical use. Drugs acting at the PCP site in the NMDA channel, including ketamine and MK801, are effective only at doses that produce behavioural disturbances including
sedation, and cognitive and behavioural changes (Willets, Balster, & Leander, 1990). In addition, administration of MK801, PCP, ketamine, AP5 and CPP have been shown to cause neurotoxic side effects consisting of vacuolisation of neuronal cytoplasm in the majority of neurons in layers 3 and 4 of the posterior cingulate and retrosplenial cerebral cortices (Olney, Labruyere & Prince, 1989; Olney, Labruyere, Wang, Wozniak, Price, & Sesma, 1991). At high doses, the toxic effects of these drugs are irreversible (Allen & Iversen, 1990). The use of drugs acting at the NMDA receptor is thus limited, both by the time window in which they have been shown to be therapeutic, by the decreased efficacy of noncompetitive antagonists during extreme membrane depolarisation, and by the potential damaging side effects. However, one approach that has received little attention is blockade of the glycine site on the NMDA receptor. This will be discussed in more detail below.

Non-NMDA antagonists

Another target for pharmacological intervention in ischemia has been the non-NMDA ionotropic receptors, as activation of AMPA and KA receptors may be required for initial NMDA receptor activation, and may also allow opening of voltage sensitive calcium channels, an alternative means of calcium influx. In addition, recent evidence suggests that the delayed neuronal death that is triggered by calcium may require intact afferent (glutamatergic) input during the period over which neuronal damage develops (Diemer, 1990). As the AMPA and KA receptors mediate normal glutamatergic transmission (Headley & Grillner, 1990), they may play a vital role in the later events leading to neuronal death in ischemia.
Recently a series of drugs that act as competitive antagonists at the KA and AMPA receptors—the quinoxaline diones—has been developed (Honore et al., 1988). Of this group of compounds, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX) has the highest affinity and selectivity for AMPA receptors, inhibits KA receptors with lower affinity, and has no activity on the NMDA and glycine recognition sites (Sheardown, Nielsen, Hansen, Jacobsen, & Honore, 1990). NBQX administered systemically 15 and 5 min before and 10 min after 5 min bilateral carotid occlusion in gerbils produced clear neuroprotection against neuronal death in CA1 assessed four days after ischemia. NBQX was also protective when administered up to 2 h after ischemia (Sheardown et al., 1990). Subsequent experiments have shown that NBQX has protective effects when administered as long as 6 h after 5 min bilateral carotid occlusion in gerbils (Sheardown, Hansen, Eskesen, Suzdak, Diemer & Honore, 1990), and 2 h after 10 min 4VO in rats (Diemer, Johansen & Jorgensen, 1990). However, NBQX administered over the first three hours following reperfusion was not protective against CA1 cell loss following 20 min 4VO in rats (Meldrum, Smith, Le Peillet, Moncada & Arvin, 1990).

One potential limit to the use of non-NMDA antagonists stems from the fact that excitatory amino acids are the major excitatory neurotransmitters in the brain, and normal excitatory synaptic transmission is mediated by the actions of these neurotransmitters at AMPA and KA receptors (Headley & Grillner, 1990). Blockade of these receptors is therefore likely to produce general inhibition of synaptic activity, and may result in unacceptable CNS depression.
Glutamate release blockers

A third pharmacological strategy that has been employed to interfere with the activation of glutamate receptors during ischemia is to prevent glutamate release. Several drugs have been developed that inhibit glutamate release \textit{in vitro}, including lamotrigine (Leach, Marsden & Miller, 1986) and 2-amino-6-trifluoromethoxy benzothiazole (riluzole). Riluzole was effective in preventing the neuronal degeneration in CA1 induced by 10 min bilateral carotid occlusion in gerbils if administered both before and after the period of ischemia (Malgouris et al., 1989). Adenosine agonists also reduce synaptic glutamate release (Dolphin & Archer, 1983), and administration of propentofylline (HWA 285) (DeLeo, Toth, Schubert, Rudolphi & Kreutzberg, 1987; DeLeo, Schubert & Kreutzberg, 1988), and 2-chloroadenosine (Evans, Swan, & Meldrum, 1987), which are potent adenosine agonists, reduces ischemic damage following 10 min carotid occlusion in gerbils and rats, respectively. However, at efficacious doses of these adenosine agonists moderate side effects including hypothermia, sedation and hypotension are seen (Dean et al., 1991) which may limit their therapeutic use.

Recently, it has been demonstrated that the noncompetitive NMDA antagonists MK801, ketamine and PCP also inhibit glutamate release during anoxia in the \textit{in vitro} hippocampal slice preparation (Lobner & Lipton, 1990). Furthermore, these noncompetitive NMDA antagonists were not able to prevent the irreversible failure of synaptic transmission in CA1 induced by anoxia unless they were administered at concentrations which were sufficient to prevent glutamate release. Therefore, one mechanism by which noncompetitive NMDA antagonists may provide protection
against transient global or forebrain ischemia *in vivo* is by attenuating glutamate release during ischemia.

**Other approaches**

Other strategies aimed at preventing the excitotoxic effects of glutamate in ischemia include the application of calcium channel antagonists, the administration of drugs which interfere with calcium-activated kinases, and the use of drugs that inhibit free radical production.

Calcium influx via voltage-operated calcium channels (VOCCs) may contribute to ischemic damage in at least two ways (Choi, 1988b). First, stimulation of VOCCs as a consequence of the depolarisation mediated by ion flux through non-NMDA and NMDA receptor linked ionophores would allow calcium influx into cells, and this may contribute to the general elevation in intracellular calcium, and subsequent calcium-mediated damage. Second, calcium influx via VOCCs into presynaptic terminals may contribute to neurotransmitter release during ischemia, thereby enhancing the propagation of injury.

At least four different types of neuronal VOCCs (L-, T-, N-, and P-types) have been differentiated on the basis of their functional properties, including voltage- and time-dependence, single channel kinetics, sensitivity to antagonists, and cellular distribution (Nowycky, Fox, & Tsien, 1985; Llinas, Sugimori, Lin & Chersky, 1989); on the basis of molecular evidence it has been suggested that there may be even greater diversity (Tsien, Ellinor & Horne, 1991). Different types of VOCCs may be important in different aspects of neuronal function (Miller, 1987). For example, L-type channels are thought to be located primarily on postsynaptic cell membranes, and their blockade
Chapter 1

by dihydropyridines (a class of compounds that acts as antagonists at L-type, but not T, N, or P-type channels) attenuates depolarisation-induced calcium influx into cell soma. In contrast, N-type channels are thought to be involved in neurotransmitter release; application of w-conotoxin—a potent antagonist of N-type, and possibly L-type, neuronal calcium channels—inhibits the evoked release of norepinephrine and serotonin from rat-brain synaptosomes (Reynolds, 1986), and of norepinephrine release from rat sympathetic neurons (Hirning et al., 1988). Dihydropyridine compounds were ineffective in attenuating evoked neurotransmitter release in these studies, suggesting calcium influx via N-type, and not L-type calcium channels is preferentially involved in transmitter release.

Calcium channel blockers have had mixed success in animal models of global ischemia (Siesjo, 1988 for review), and much of their therapeutic efficacy is thought to be due to their ability to increase cerebral blood flow following ischemia. However, as the vast majority of these studies have examined the protective abilities of dihydropyridines, the lack of consistent effects of calcium channel blockers in ischemia may be attributable to the selective antagonism of L-type channels, which would be expected to attenuate postsynaptic calcium accumulation, but may not affect neurotransmitter release. A recent study showed that w-conotoxin (which blocks both N- and L-type channels) administered immediately following and up to 90 min after ischemia attenuated the CA1 damage induced by 15 min 4VO in rats (Valentino et al., 1991). This finding suggests that blockade of N-type calcium channels, and the subsequent inhibition of neurotransmitter release, may offer more protection in ischemia than inhibition of calcium flux into cells via L-type channels, which is just one of many possible routes by which calcium can enter postsynaptic neurons.
Some protective effects have been reported using drugs that interfere with calcium-mediated processes within neurons. For example, postischemic treatment of di-calciphor decreases neuronal death in the hippocampus following 20 min bilateral carotid occlusion in gerbils (Von Lubitz, Devlin, Kalenak, Lin, Matesic, & McKenzie, 1991). This compound prevents the degradation of spectrin and MAP2 which is known to be mediated by the calcium-activated protease calpain I (Matesic, Devlin, Kalenac, McKenzie, von Lubitz, & Lin, 1991). Similar protective effects have been found for other calpain inhibitors administered after ischemia induced by 4VO in rats (Bartus, Dean, Eveleth, Lutz, Harris, & Powers, 1991).

Free radical scavengers are also effective in preventing ischemia-induced cell loss in some preparations, although in most cases the protective effects are only partial (for review see Schmidley, 1990). As mentioned above, free radicals may contribute to glutamate release during ischemia, so one means by which free radical scavengers may attenuate ischemic damage is by inhibiting release of glutamate. They would also decrease other neurodegenerative processes mediated by free radical species. One reason that free radical scavengers offer only partial protection is that free radical production is just one of many mechanisms of ischemia-induced cell death.

Summary

In conclusion, each of the strategies that have been used to interfere with glutamatergic activity and the effects of subsequent calcium accumulation during ischemia and the postischemic period have been partially successful in attenuating neuronal damage in in vivo animal models of global or forebrain ischemia. Pharmacological intervention early in ischemia may be more protective than attempts to
inhibit later events, as it is likely that early accumulation of intracellular calcium stimulated by glutamate receptor activation is responsible for triggering many processes which contribute to the eventual cell death. Drugs which prevent only a subset of these processes can at best only attenuate or delay the damage. However, it is apparent that the activation of glutamate receptors may be important both in stimulating the initial calcium accumulation, and in mediating some aspects of delayed neuronal death, as blockade of AMPA receptors as long as 6 h after ischemia prevents ischemic damage. A prominent hypothesis is that the initial calcium influx sensitises cells to subsequent glutamate release, and that normal synaptic input following this sensitization may induce lethal damage (Diemer et al., 1990).

Despite previous mixed effects of NMDA antagonists, the most useful strategy may be to prevent the initial intracellular accumulation of calcium stimulated by NMDA receptor activation. One approach that has received little attention in this context is the use of glycine site antagonists. As discussed above, agonist binding at the strychnine-insensitive glycine binding site associated with the NMDA receptor has been shown to potentiate NMDA receptor-mediated responses (Johnson & Ascher, 1987), and it has been proposed that glycine acts as a co-agonist at the NMDA receptor channel complex (Kleckner & Dingledine, 1990). In addition, glycine has been shown to potentiate NMDA-mediated toxicity in vitro (Patel, Zinkland, Thompson, Keirth & Salama, 1990). Therefore, prevention of glycine binding may prevent ischemia-induced calcium influx via the NMDA receptor. Recently, it has been shown that kynurenic acid, an endogenous tryptophan metabolite, and several kynurenic acid derivatives act as antagonists at this site (Kessler, Terramani, Lynch, & Baudry, 1989). A structurally related compound, 7-Chlorokynurenic acid, has 70-fold greater affinity for the glycine
site than kynurenic acid, and also demonstrates high selectivity for this site (Kemp et al., 1988). The potential of this selective glycine site antagonist to protect neurons against ischemic damage is suggested by a preliminary report of its neuroprotective effects in gerbils in vivo (Patel, Ross, Duncan, Asif, Salama, & Valerio, 1989). The neuroprotective efficacy of 7-Chlorokynurenic acid in ischemia will be investigated further in his thesis.

THE PRESENT STUDY

As discussed above, the development of animal models of ischemia has been crucial to our understanding of the mechanisms of ischemic neuronal damage, and to the development and assessment of treatments which may attenuate ischemic damage in vivo. Recently, animal models of transient global or forebrain ischemia have also been instrumental in the investigation and characterisation of the impairments in learning and memory that accompany ischemic brain damage. In rats, deficits in performance on a variety of spatial working memory tasks have been demonstrated following transient forebrain ischemia, and in monkeys transient forebrain ischemia results in deficits on DNMS, a recognition task that is analogous to tasks on which human amnesic patients perform badly.

These descriptions of impairments in learning and memory following ischemia in animals are important for two reasons: first, they provide animal models of ischemia-induced amnesia in humans and thereby enable investigation into the specific patterns of ischemia-induced brain damage that underlie the memory impairments. This, in turn, may have importance for basic studies of memory. Second, they provide
a behavioural measure of outcome for treatments designed to protect the brain against ischemic damage.

The utility of animal models of ischemia-induced amnesia is enhanced if the behavioural measures used can be compared easily to those used for humans. In particular, the use of comparable tasks allows inferences to be made across species concerning the neuropathological basis of ischemia-induced memory impairments, and the therapeutic effects of pharmacological intervention. In this context, the behavioural findings from the present rat models are difficult to relate to ischemia-induced memory deficits in monkeys and humans, as the tasks used cannot be equated easily.

The experiments reported in this thesis are designed first to extend the characterisation of the behavioural effects of transient forebrain ischemia in rats to non-spatial memory tasks that are analogous to those on which humans and monkeys are impaired, and second, to provide a preliminary assessment of the neuroprotective efficacy of a drug that blocks the glycine site associated with the NMDA receptor.

In Chapter 2, the pattern of damage induced by 20 min transient forebrain ischemia induced by bilateral carotid occlusion combined with hemorrhagic hypotension to 30 mmHg in rats is assessed. Such an analysis is essential if inferences are to be drawn concerning the anatomical basis of learning and memory deficits in rats, and for the application of these findings to humans. In the experiments in Chapter 3, the performance of rats on nonspatial recognition tasks is characterised, and the relationship between ischemia-induced recognition memory deficits and brain damage assessed. The findings of these studies are related to ischemia-induced memory impairments in monkeys and humans. In the Experiments in Chapter 4, the protective properties of 7-Chlorokynurenic acid—a potent antagonist at the glycine site associated
with the NMDA receptor—are assessed using histological and neurobehavioural measures of outcome.
CHAPTER 2: GENERAL METHODS EMPLOYED TO INDUCE TRANSIENT FOREBRAIN ISCHEMIA IN RATS, AND HISTOLOGICAL ANALYSIS OF THE ISCHEMIC DAMAGE

INTRODUCTION

As described in the General Introduction, the development of animal models of ischemia-induced amnesia is vital to the analysis of the functional consequences of ischemic damage, and to testing the behavioural efficacy of potentially therapeutic drugs. One important goal of research investigating ischemia-induced amnesia is to delineate the neuropathological changes that underlie the memory impairments. To achieve this goal it is necessary to characterise both the behavioural and the anatomical consequences of ischemia, and to examine the relationship between them. As discussed in the previous chapter, several animal models of ischemia have been used in the investigation of ischemia-induced memory deficits. These models differ along several dimensions including species, method of induction, duration of ischemia, anesthetic, and temperature of the animal, many of which can affect the extent and location of ischemia-induced cell loss. Therefore, in order to draw conclusions as to the anatomical substrates of ischemia-induced memory deficits in animal models it is essential to assess the location and extent of ischemic cell loss for each model of ischemia, and not to generalise anatomical or behavioural results obtained across studies in which different methods were used to induce ischemia. Accordingly, this chapter provides a description of the method used to induce transient forebrain ischemia in rats in each of the experiments reported in this thesis, and the pattern of ischemic cell loss that is produced.
The method used to induce ischemia in each of the experiments combined 20-min bilateral carotid occlusion with hypotension. This method was originally modified by Mudrick, Leung, Baimbridge & Miller (1988) from the method described by Smith et al. (1984). This model of transient forebrain ischemia has been reported to cause selective severe damage to CA1 hippocampal pyramidal neurons (Mudrick et al., 1988). Long term histological and immunohistochemical studies have shown that at 6 months after ischemia induced by this method, cell loss within the hippocampus is largely restricted to the CA1 region, and involves primarily pyramidal neurons. There is also some loss of somatostatin-immunoreactivity, indicating cell loss in the hilus of the dentate gyrus (Mudrick & Baimbridge, 1989). We chose to use this model of transient forebrain ischemia because the pattern of hippocampal damage it produces is similar to the pattern reported in the clinical case R.B., whose lesion was restricted to the CA1 cell field, and did not include the CA3 or subiculum (Zola-Morgan et al., 1989). Other models, including the four vessel occlusion model often used in experiments investigating memory performance following ischemia (e.g Davis & Volpe, 1990), typically produce more extensive hippocampal damage; CA2 and CA3 fields, as well as the subiculum, often suffer cell loss. The four vessel occlusion model of ischemia also produces consistent extrahippocampal damage, particularly within the striatum (Davis & Volpe, 1991). As lesions of the striatum alone can produce deficits on some memory-related tasks (Packard, Hirsch & White, 1989), it is possible that striatal damage may have contributed to some of the memory deficits seen following four vessel occlusion in previous experiments (e.g. Davis & Volpe). As striatal damage is not a consistent feature in ischemia in humans (Zola-Morgan, Squire, & Amaral, 1989), it is more appropriate to use a model that does not produce striatal cell loss.
In this experiment, the cell loss produced by this bilateral carotid occlusion model of transient forebrain ischemia was quantified several weeks following ischemia, both within the hippocampus, and in several extrahippocampal brain areas, including the striatum, mammillary nuclei, entorhinal cortex and perirhinal cortex. The aims were twofold: first, to verify that this model of ischemia produced a pattern of hippocampal cell loss similar to that seen in humans; and second, to determine whether this model of ischemia produced damage to extrahippocampal brain areas that are thought to be involved in memory.

**METHOD**

**Subjects**

The subjects were 21 male Wistar rats (Charles River, Quebec) that weighed 300-325g at the time of surgery. They were housed in group cages with continuous access to water and rat chow under a 12:12 hour dark:light cycle with light onset at 8:00 am. Following surgery until they could eat solid rat chow, the rats also had access to wet mash (rat chow soaked in water).

**Surgery**

The experimental rats were treated with atropine sulphate (1.0 mg/kg i.p.) to decrease respiratory tract secretions associated with anesthesia and ischemia. 20 min later they were anesthetised with sodium pentobarbital (65 mg/kg i.p.). Both common carotid arteries were isolated by blunt dissection, and loosely encircled with 5-0 silk ligatures. The femoral artery was cannulated with PE50 tubing connected to a saline-
primed reservoir via a pressure transducer (Electromedics Inc.). The whole system was calibrated with a mercury manometer system, and arterial blood pressure was recorded on a Y-T single channel chart recorder (Kipp & Zoner, Johns Sci. Inc.). The experimental rats initially received a 0.1 ml bolus of heparinized saline (100 units) via the arterial cannula, and were treated periodically with small volumes (0.1 ml) of more dilute heparinized saline (20 units/ml) to prevent the cannula becoming occluded. To induce ischemia, first the rats were allowed to hemorrhage via the arterial cannula until the mean arterial blood pressure dropped to 30 mm Hg (approximately 15 min). The shed blood was collected in the saline-primed reservoir. As soon as this level of hypotension was reached, the silk ligatures were pulled up gently to expose the carotid arteries, and the arteries were occluded with atraumatic arterial clamps. After 20-min of bilateral carotid occlusion, during which the mean arterial pressure was maintained at 30 mm Hg by withdrawing or reinfusing blood as necessary, the clamps were removed and the collected blood reinfused over approximately 15-min by applying 200 mm Hg back pressure to the reservoir. Mean arterial pressure quickly returned to previous values following reinfusion of the blood, at which point the femoral artery was tied off, the cannula removed, and the wounds sutured.

Rats were allowed to breathe spontaneously, and their core temperature was maintained at 36°C with a temperature controlled water heating pad (American Hospital Supplies, McGraw Park, UL) throughout the course of surgery and for 30-min following reinfusion. Sham-ischemia control rats were subjected to the same procedures as the ischemic rats except they were not subjected to hemorrhaging and carotid artery occlusion.
Following surgery each rat was placed in a plastic cage in a recovery room and monitored closely for three days. During this period the ambient illumination and noise levels were kept as low as possible, to minimise the occurrence of motor seizures. It had previously been found that 24 to 72-h following transient forebrain ischemia induced using this procedure approximately 70% of rats have tonic-clonic seizures and of those that display seizure activity approximately 50% die (Damsma, Boisvert, Mudrick, Wenkstern, & Fibiger, 1990). We anticipated a similar occurrence of seizures, and wanted to decrease the number of rats dying as a result of the seizures. Two procedures were employed to minimise mortality following ischemia; rats were aspirated if they appeared to have problems breathing, and some rats also received supplemental doses of atropine sulphate to decrease respiratory tract secretions. In addition, diazepam (10 mg/kg i.p.) was administered to all rats to decrease the incidence of behavioural seizures. Previous studies have shown that neither the occurrence of behavioural seizures, nor the administration of diazepam significantly affects the pattern or magnitude of cell damage following ischemia (Mudrick, unpublished observations).

**Neuropathological Assessment**

In order to assess the pathological changes in the brain resulting from ischemia, 10 weeks following surgery rats were anesthetised with sodium pentobarbital (100 mg/kg ip) and perfused transcardially with 10% formalin in 0.05% phosphate buffer (pH 7.4). Their brains were removed immediately and stored overnight in buffered formalin. Later, the brains were processed in graded ethanols and xylene and embedded in paraffin. Coronal sections were cut at 10 um from the rostral end of the striatum to
Chapter 2

the caudal end of the hippocampal formation; every 10th section was mounted and stained for nissyl substance with 0.1% cresyl violet. Quantification of neuronal damage was performed by direct visual counting of viable neurons using a light microscope at 40x power. In the hippocampus, cells were quantified in six different sections for each rat. These six sections were distributed evenly along the septo-temporal axis of the hippocampal formation (see Figure 5). At each of the six hippocampal levels, all of the pyramidal neurons in the CA1, CA2-CA3\(^1\) cell fields, the polymorphic cells of the dentate hilus\(^2\); the granule cells of the dentate gyrus; and the dentate hilar cells were counted. These hippocampal subdivisions are illustrated in Figure 2. For each of these regions the cell counts were averaged across the right and left hippocampi to generate a mean cell count at each of the six levels for each rat. CA1, CA2-3, CA4 and dentate gyrus granule cell counts were expressed as number of cells per unit length of the cell field (cells/125 um), whereas hilar cell counts were expressed as number of cells per unit area (cells/100 um\(^2\)). Percent cell loss for each ischemic rat was calculated by comparing the mean cell count at a given level with the average mean cell count at the same level for the sham ischemia control rats.

\(^1\)The transition from CA2 to CA3 was not detectable, so cell counts for CA2 and CA3 were combined for each rat.

\(^2\)The area defined here as CA4 refers to the dispersed pyramidal cell band between the two blades of the dentate gyrus. These cells are often included as part of the CA3 cell field (Schmidt-Kastner & Freund, 1991), and are commonly referred to as the polymorphic cells of the dentate hilus. However, for the purposes of cell quantification in the present experiment, it was useful to distinguish between these cells and the undispersed CA3 pyramidal cell band beyond the dentate gyrus.
Figure 2. Schematic diagram of a coronal section of the hippocampal formation, illustrating the subdivisions of the hippocampus in which cells were quantified. (CA1, CA2-3 and CA4 mark the pyramidal cell fields; DG-G marks dentate gyrus granule cells; DG-H marks hilus of the dentate gyrus.)
Cell counts were also performed in the striatum, the mammillary bodies, and in entorhinal and perirhinal cortices. For each of these structures, cells were quantified in a single section: In the striatum, the number of cells in four quadrants, corresponding to dorso-lateral, dorso-medial, ventro-lateral and ventro-medial, were counted at the level of the anterior commissure (Bregma -0.3 mm). The number of neurons per unit area (cells/100 \text{ um}^2) was calculated for each quadrant. Neurons in the mammillary nuclei were quantified at the level of the mammillary peduncle (Bregma -4.8 mm), and entorhinal and perirhinal neurons were counted at Bregma -5.3 mm. These cell counts were also expressed as number of cells per unit area. Percent cell loss for ischemic rats in each of the extrahippocampal structures was calculated in the same way as for hippocampal cell counts. The rationale for quantifying neurons in these extrahippocampal regions was that they have been reported either to be particularly susceptible to transient forebrain ischemia (striatum, particularly the dorso-lateral portion; Pulsinelli, 1985), and/or have been implicated in amnesic syndromes (mammillary nuclei, entorhinal and perirhinal cortices; Zola-Morgan & Squire, 1990).

**RESULTS**

**Mortality**

9 rats in the ischemia group (47.37%) exhibited tonic-clonic seizures within three days of surgery and of these 5 (55.56%) subsequently died, despite frequent aspiration and maintenance doses of diazepam and atropine sulphate. This left 10 rats in the ischemia group and 6 rats in the sham ischemia group.
Neuropathology

The results of the cell counts in the pyramidal cell layers of the CA1, CA2-3, and CA4 hippocampal cell fields, in the granule cell layer and hilus of the dentate gyrus, in the striatum at the level of the anterior commissure, in the mammillary nuclei, and in the entorhinal and perirhinal cortices are summarised in Figure 3. Differences between the cell counts (expressed as mean cells for a given rat in a given brain area) of the two groups (ischemia and sham ischemia) across all areas in which neurons were quantified were analysed using a repeated measures ANOVA, with group as a between factor and brain area as a repeated measures factor. The sphericity test indicated that the data did not satisfy the assumption of circularity. Accordingly, degrees of freedom were adjusted using the Greenhouse-Geisser Epsilon. No overall significant difference was found between the two groups ($F=1.39, df=1, p > 0.05$); however, there was a significant group x brain area interaction ($F=16.38, p < 0.01$). Post hoc analyses (Tukey, with significance levels corrected for multiple comparisons using a Bonferroni procedure) revealed that ischemic rats had significantly fewer CA1 pyramidal neurons (averaged across all six levels of the hippocampus) ($p < 0.01$) than control rats, but they suffered no significant cell loss in any of the other brain areas in which cells were quantified.

Averaged across the entire septo-temporal extent of the hippocampus, the degree of CA1 cell loss in the ischemic rats ranged from 20% for the least affected animal to 87% for the most affected. Figure 4 illustrates the cell loss in the CA1 of one ischemic rat compared to the intact CA1 of a control rat at one level--level 2. In order to analyze the pattern of CA1 cell loss along the septo-temporal extent of the hippocampus, a second repeated measures ANOVA was conducted, using only the CA1 cell count data.
Figure 3. Histograms of the mean cell counts for sham-ischemia control rats (solid bars) and ischemic rats (hatched bars) expressed as a percentage of the mean cell counts for sham-ischemia control rats in each of the following areas: pyramidal neurons in CA1, CA2-3, and CA4, dentate granule cells (DG-G), dentate hilar neurons (DG-H), striatal neurons (STRIAT), entorhinal cortex neurons (ENT), perirhinal cortex neurons (PERI), and mammillary nuclei neurons (MAMM). Vertical bars show standard errors, ** denotes significant cell loss at $p < 0.01$. 
Figure 4. Top: Photomicrographs of the hippocampal formation at level 2 of the hippocampus from A a representative rat in the sham-ischemia control group illustrating the normal cell density and distribution, and from B a rat in the ischemia group illustrating the degeneration of CA1 pyramidal neurons. Arrowheads depict the CA1 region. Bottom: Photomicrographs of part of the CA1 cell field from the same control rat C and ischemic rat D, illustrating a substantial decrease in the number of viable pyramidal cell bodies following ischemia. Boxes on A and B indicate the region of CA1 from which the photomicrographs in C and D were taken.
In this analysis, the between subjects factor was groups, and the repeated measures factor was the septo-temporal level of the hippocampus (levels 1 to 6). There was a significant main effect for group \( (F=23.99, \text{df}=1, p<0.01) \), a significant effect of level \( (F=4.10, \text{df}=5, p<0.01) \), and a significant group x level interaction \( (F=5.23, \text{df}=5, p<0.01) \). Post-hoc comparisons (Tukey) showed that ischemic rats had significantly fewer CA1 pyramidal neurons than controls at all levels \( (ps<0.01) \) except at the most temporal level counted (Level 6)--see Figure 5.

Inspection by light microscopy of areas of the brain that were not subjected to formal cell counts (e.g. various diencephalic nuclei, the amygdala and various cortical areas) revealed no observable damage to these structures.

**DISCUSSION**

Transient forebrain ischemia induced by a combination of 20 min bilateral carotid occlusion and hemorrhagic hypotension to 30 mm Hg resulted in extensive loss of pyramidal cells in the CA1 field of the hippocampus, in the absence of any significant visible cell loss in other hippocampal cell fields, or in the striatum, mammillary nuclei, entorhinal cortex or perirhinal cortex. In addition, no damage was detected in brain areas in which cells were not quantified. This pattern of cell loss is consistent with previous descriptions of the hippocampal cell loss produced by this model of ischemia (Mudrick et al., 1989); the primary lesion is of CA1 pyramidal neurons, and there is no loss of pyramidal cells in CA2-4, or of dentate granule cells. However, in the present experiment there was no evidence of hilar neuron loss, whereas previous immunocytochemical studies have shown extensive loss of somatostatin-containing neurons in the hilus of the dentate gyrus (Mudrick et al.,...
Figure 5. Density and distribution of ischemia-induced CA1 cell loss from the septal end (level 1) to the temporal end (level 6) of the hippocampal formation for the rats in Chapter 2. The percentage of CA1 cell loss at each level, and the mean for the 6 levels combined (X) are shown as horizontal bars (mean ± SE). The corresponding septo-temporal levels are depicted on the right. ** denotes significant cell loss at \( p < 0.01 \). The drawings of the hippocampus are adapted from Auer, Jensen, & Whishaw (1989).
The lack of detectable hilar neuron loss is particularly surprising given the well-documented vulnerability of these cells to ischemia (Schmidt-Kastner & Freund, 1991). One possible explanation for our findings is that the loss of a subset of hilar neurons (i.e., the vulnerable somatostatin-containing neurons) may not have been detected using cell counts that did not distinguish between neuronal types.

The extent of CA1 pyramidal cell loss varied along the long axis of the hippocampus; consistent with previous findings (Auer et al., 1989; Ashton et al., 1989; Smith et al., 1984a) there was greater neuronal loss in the septal hippocampus than at its caudal pole. This pattern may be due in part to vascular factors (Schmidt-Kastner & Freund, 1991); there may be some residual blood supply to the temporal hippocampus via the unoccluded vertebro-basilar arterial system during the period of ischemia, or differences in post-ischemic blood flow to septal and caudal regions of the hippocampus may contribute to the gradient of damage.

The pattern and extent of cell loss within the hippocampus was similar to that produced by other models of ischemia in rats, but there was less extrahippocampal damage than has been reported following both 30 min four vessel occlusion (Davis & Volpe, 1990; Pulsinelli & Brierley, 1979), and 10.5 min bilateral carotid occlusion combined with hypotension to 50 mm Hg (Rod, Whishaw & Auer, 1990)—both methods produced significant striatal cell loss. The use of a barbiturate anesthetic may account for the striatal-cell sparing in the present experiment. Pentobarbitol decreases dopamine efflux during ischemia (Bhardwaj, Brannan, & Weinberger, 1990), and dopamine has been implicated in the pathophysiology of ischemia-induced striatal cell loss (Globus, Ginsberg, Busto & Dietrich, 1987).
Although the only detectable cell loss was of CA1 pyramidal neurons, ischemia may have produced pathological changes in hippocampal and extrahippocampal areas that were not observable with the histological techniques we used. As discussed above, loss of a small specific subset of neurons within one of the brain areas may not have been detected. It may be possible to detect such cell loss using immunohistochemical techniques. In addition, ischemia-induced changes in the biophysical properties of neurons would not be detected with cell counts, but may result in functional changes to brain areas that appear to be intact.

The correspondence between the histological changes induced by this bilateral carotid occlusion model of transient forebrain ischemia in rats, and the pattern of damage seen in the ischemia-induced amnesic patient R.B. (Zola-Morgan et al, 1989) is consistent with this being a useful animal model of ischemia with which to investigate ischemia-induced amnesia. However, given the large degree of inter-animal variability in the extent of CA1 cell loss, it will be necessary to quantify the CA1 cell loss in each animal if the relationship between ischemia-induced cell loss and ischemia-induced deficits in learning and memory are to be assessed.
CHAPTER 3: ANALYSES OF THE PERFORMANCE OF RATS ON NONSPATIAL MEMORY TASKS FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA

As described in the General Introduction, humans (Cummings et al., 1984; Volpe & Hirst, 1983; Volpe & Petito, 1985; Zola-Morgan et al., 1986), monkeys (Bachevalier & Mishkin, 1989; Zola-Morgan et al., 1992) and rats (Volpe et al., 1984, 1988, Davis et al., 1986; Davis & Volpe, 1990; Auer et al., 1989) suffering transient global ischemic insults have all shown deficits on memory tasks. The descriptions of memory deficits following ischemia in monkeys and rats have provided useful information concerning both the nature of the memory impairments, and the underlying neuropathological changes that are produced by ischemia. Transient cerebral ischemia in monkeys produces permanent deficits on a nonspatial object recognition task—the nonrecurring-items delayed nonmatching-to-sample task (DNMS) (Bachevalier & Mishkin, 1989; Zola-Morgan, Squire, Rempel, Clower, & Amaral, 1992). These object recognition memory deficits are associated with significant loss of CA1 and CA2 hippocampal pyramidal neurons and of cells in the hilus of the dentate gyrus, with little or no significant damage to extrahippocampal structures (Bachevalier & Mishkin, 1989; Zola-Morgan et al, 1992). Both the behavioural and the neuropathological profiles of ischemic monkeys are similar to those of humans with ischemia-induced amnesia; ischemic patients have been shown to perform poorly on DNMS (Squire, Zola-Morgan, & Chen, 1988), and in the case of R.B., the memory impairments were thought to be mediated by circumscribed loss of CA1 pyramidal neurons (Zola-Morgan et al, 1989).

Similarly, transient forebrain ischemia in rats results in deficits on spatial memory tasks (e.g. Auer, Jensen & Whishaw, 1990; Davis & Volpe, 1990; Jaspers et
al, 1990). In general, ischemia induced either by 4-vessel occlusion or by bilateral carotid occlusion results on deficits on spatial working memory tasks and, to a lesser degree, on spatial reference memory tasks. The predominant lesion in these rat models of ischemia involves CA1 hippocampal pyramidal neurons. Therefore, the results of these studies are consistent with the hypothesis that hippocampal damage is responsible for ischemia-induced memory deficits. However, the relation of ischemia-induced memory deficits in rats to those in monkeys and humans is difficult to assess given that rat studies have focused on spatial tasks and monkey and human studies have focused on object recognition tasks. The use of non-spatial tasks for rats that are analogous to those used to assess humans and monkeys would enable comparisons between the species, and may also provide a more appropriate rat model for assessing the effects of ischemic brain damage.

The aims of the experiments in this chapter are to assess the performance of rats with ischemic damage on non-spatial recognition memory tasks, and to investigate the relation between ischemia-induced brain damage and behavioural impairment on these tasks. This description will allow comparisons between the effects of ischemia in rats, humans and monkeys, and may also provide a useful rat model for the behavioural assessment of the effects of potential anti-ischemic drugs.

**Experiment 3.1: One-Trial Object Recognition**

**INTRODUCTION**

This experiment examined the performance of rats on a simple one trial object-recognition task four weeks following transient forebrain ischemia. This task,
developed by Ennaceur & Delacour (1988), is based on the differential exploration of novel and familiar objects, and takes advantage of rats' natural predisposition to explore novel objects (Berlyne, 1960). In this task, rats are exposed to two identical objects (samples). Some time later they are exposed to two dissimilar objects; one is familiar (the same as the samples), and the other is new. Normal rats discriminate between objects on the basis of novelty; that is, they tend to spend more time exploring the novel object than the object to which they have recently been exposed. It has been argued that this task is a non-spatial memory task similar to object-recognition tasks (such as DNMS) used to test monkeys and humans; object recognition can be measured by the difference in exploration time of the new and the familiar objects. Furthermore, the ability of rats to discriminate between the novel and sample object decreases as the time interval between exposure to the sample objects, and to the sample and novel object increases. This is similar to the decrease in object-recognition by normal monkeys seen on the DNMS task when the retention delay is increased. Ennaceur and Delacour (1988) also claim that this task can be considered a pure working memory task; there are no reference memory components to the task, as it is based on the spontaneous behaviour of rats.

Given the parallels between this task and non-spatial object-recognition tasks on which monkeys and humans perform poorly following ischemia, this task may represent a useful task for assessing ischemia-induced memory deficits in rats. Accordingly, the performance of ischemic rats and sham-ischemia control rats on this one-trial object recognition tasks was assessed, and the relationship between ischemia-induced cell loss and behavioural changes determined.
METHOD

Subjects
The subjects were 28 male Wistar rats (Charles River, Quebec) that weighed 300-350g at the time of surgery. None had been used in any previous experiments. However, the anatomical data from 8 of them (4 ischemics and 4 controls) were used in the analyses described in Chapter 2. The rats were housed in group cages under a 12/12 h light/dark cycle, and had unlimited access to food and water.

Apparatus
The testing apparatus consisted of an opaque cylindrical Plexiglass chamber (radius 25 cm, depth 50 cm). The top of the chamber was removed, and a video-camera was positioned above the chamber. The test stimuli were a collection of wooden objects; there were four identical exemplars of each of two objects—pyramids and cylinders—which had the same surface area, volume and colour.

Surgical Procedure
20 min transient forebrain ischemia was induced in 16 rats according to the procedures outlined in Chapter 2 above. 12 sham-ischemia control rats underwent the same procedures with the exception of hemorrhaging and carotid occlusion. Postsurgical care of all animals was conducted as described in Chapter 2. All rats were allowed to recover for 35 days before behavioural testing began.
Neuropathological Assessment

After behavioural testing, the rats were sacrificed, and their brains prepared for histological evaluation following the procedures described in Chapter 2. CA1 pyramidal neurons were quantified for each rat in 6 hippocampal sections distributed along the septo-temporal axis of the hippocampus, as described in Chapter 2.

Behavioural Procedure

The testing procedures were similar to those described by Ennacour & Delacour (1988). Beginning 30 days after surgery, each rat was habituated to the testing chamber on three consecutive days, and received a single test on the fourth day. On the three habituation days, each rat was placed into the testing chamber for 15 min and allowed to explore. On the test day, each rat received one trial consisting of three phases: exposure, retention interval, and test. In the exposure phase two identical objects (either two cylinders or two pyramids) were positioned in the chamber. The rat was placed in the chamber and allowed to explore for 3 min, during which its behaviour was videotaped. The rat was then removed from the apparatus to a holding cage. At this time the two objects were removed from the chamber and replaced by two new objects. One was identical to the two objects present during the exposure phase; the other—the novel object—was an exemplar of the type to which the rat had not previously been exposed. After 1 min (the retention interval) the rat was replaced in the test chamber and allowed to explore for 3 min. Again, its behaviour was videotaped. At the end of the 3-min exploration period, the rat was removed from the test chamber and returned to its home cage. Before the next rat was tested the testing chamber was wiped out, and the objects were rinsed in a dilute bleach solution. The object type (cylinders
versus pyramids) that was present during the exposure phase was counterbalanced for each group.

The amount of time each rat spent investigating each object in the exploration phase and in the test phase were measured from the videotapes. A rat's behaviour was identified as investigation when the rat directed its nose towards the object at a distance <2cm, and/or touched the object with its nose or vibrissae. Three variables were considered: the initial exploration period, defined as the total time spent exploring either of the two objects in the exposure phase; the test exploration period, defined as the total time spent exploring either of the two objects in the test phase; and the discrimination ratio, defined as the ratio of time spent exploring the novel object to the total time spent exploring both objects in test phase. The initial exploration and test exploration measures were used to ensure that baseline exploration durations did not differ between control and ischemic rats. The discrimination ratio was used as a measure of memory for the sample object.

RESULTS

Neuropathology

Four rats in the ischemic group died following surgery: this left 12 ischemic rats and 12 sham-ischemia control rats. A repeated measures ANOVA with group as the between factor, and level of hippocampus as the repeated measures factor showed that ischemic rats had significant bilateral loss of CA1 hippocampal pyramidal cells ($F=152.64$, $df=1$, $p<0.01$). The amount of CA1 hippocampal pyramidal cell damage sustained by ischemic rats ranged from 45.3% to 90.9% averaged across the 6 septo-
temporal levels. There was also a significant effect of level \((F=15.93, \text{df}=5, p<0.01)\) and a significant group x level interaction \((F=20.06, \text{df}=5, p<0.01)\). Post hoc tests (Tukey) showed that ischemic rats had significantly fewer remaining CA1 cells than control rats at all 6 levels \((\text{Levels 1-5, } p<0.01; \text{Level 6, } p<0.05)\) (see Figure 6).

**Behaviour**

There were no significant differences between ischemic and control rats in the duration of initial exploration of the sample objects in the exposure phase \((t=0.73; \text{df}=22, p>0.05)\), or in the total time spent exploring the objects during the test phase \((t=0.68; \text{df}=22, p>0.05)\) (see Figure 7a). On the test trial the discrimination ratio for control rats was significantly greater than for ischemic rats \((t=2.55; \text{df}=22, p<0.05)\) (see Figure 7b). That is, control rats spent a greater proportion of the object exploration time investigating the novel object than did ischemic rats. Control rats also spent significantly more time investigating the novel object than the sample object in the test phase \((\text{one-way within subject ANOVA, } F=7.40; \text{df}=11, p<0.05)\), whereas there was no significant difference between the times spent investigating the two objects for ischemic rats \((F=0.54; \text{df}=11, p>0.05)\).

In order to assess the relation between the neuropathological changes and the performance deficits induced by ischemia, the correlation between the mean number of CA1 cells per 125 \(\text{um}\) averaged across all six levels of the hippocampus, and the discrimination ratio was calculated. There was a significant correlation between the CA1 pyramidal cells remaining and the discrimination ratio \((r=0.5557, \text{df}=23, p<0.05)\). The rats with the fewest CA1 pyramidal neurons had the lowest
Figure 6. Density and distribution of ischemia-induced CA1 cell loss from the septal end (level 1) to the temporal end (level 6) of the hippocampal formation for the rats in Experiment 3.1. The percentage of CA1 cell loss at each level, and the mean for the 6 levels combined (X) are shown as horizontal bars (mean ± SE). The corresponding septo-temporal levels are depicted on the right. ** denotes significant cell loss at p < 0.01; ** denotes significant cell loss at p < 0.05. The drawings of the hippocampus are adapted from Auer, Jensen, & Whishaw (1989).
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Figure 7. A: Total duration of exploration of both objects in the exploration phase, and in the test phase, by sham-ischemia control rats (hatched bars) and ischemic rats (open bars). B: Discrimination ratios (duration of exploration of novel object : duration of exploration of novel and sample objects objects in the test phase) of sham-ischemia control rats (hatched bars) and ischemic rats (open bars). Vertical bars show standard errors.
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A

EXPLORATION DURATION (s)

CONTROL

ISCHEMIC

EXPLORATION TEST

B

DISCRIMINATION RATIO

DISCRIMINATION
discrimination ratio, indicating that they had the most severely disrupted behaviour. This relationship is illustrated in Figure 8.

DISCUSSION

Transient forebrain ischemia disrupted the behaviour of rats in this object-exploration paradigm. Sham-ischemia control rats discriminated between two objects during the test phase, on the basis of novelty, and spent significantly more time investigating a novel object than an object they had seen previously. In contrast, ischemic rats spent as much time exploring each of the objects during the test phase, indicating that they did not discriminate between the objects on the basis of novelty.

The ability to discriminate between objects on the basis of novelty can be attributed to at least two different processes. One is the ability to categorize an object as novel based on remembering the sample object seen during the exposure phase. The second concerns processes underlying stimulus preference. The results of this experiment can therefore be interpreted in several ways. The performance of ischemic rats may reflect a memory impairment; if rats are unable to remember the sample object from the exposure phase, both objects available during the test phase would be similarly "novel" to the rat, and they would explore both objects to the same extent. Alternatively, the performance of ischemic rats may reflect a disruption in their normal disposition to explore novel objects. It has been shown that lesions of the perforant path, which may be functionally equivalent to hippocampal lesions, significantly decrease the exploration of novel objects by rats (Myhrer, 1988). Alternative explanations for the results must also be considered. For example, ischemic rats may have had a sensory deficit and therefore have been unable to discriminate the sensory
Figure 8. Number of viable CA1 pyramidal neurons for individual rats plotted against the discrimination ratio (duration of exploration of novel object : duration of exploration of novel and sample objects in the test phase) for sham ischemia control rats (solid circles) and ischemic rats (open circles). The lines show the linear regression between the two variables.
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![Graph showing the relationship between CA1 cells and discrimination ratio. The graph includes a scatter plot with a regression line, indicating a positive correlation. The correlation coefficient is r=0.56; p<0.01.](image)
features of the two objects. Alternatively, ischemic rats may have been more
susceptible to distraction than control rats. However, this possibility is incompatible
with the finding that the total duration of exploration in both the exposure phase and the
test phase was the same for both groups. Therefore, although a memory deficit would
be consistent with the working memory deficits seen in rats following ischemia (e.g.
Davis & Volpe, 1990; Auer et al., 1989), the precise nature of the deficit cannot be
confirmed.

The pattern of CA1 pyramidal cell loss in the ischemic rats is similar to that
found in Chapter 2; temporal levels of the hippocampus were less severely damaged
than septal levels, and there was considerable between-animal variability in the extent
of cell loss. Although other hippocampal cell fields and extrahippocampal brain
structures were not subjected to formal cell counts, they appeared normal when
examined by light microscopy. Taken together with the quantitative histological
analysis of hippocampal and extrahippocampal cell loss typically produced by this
model of ischemia (see Chapter 2), these findings suggest that the cell loss was
restricted largely to the CA1 region of the hippocampus. Therefore, it is likely that the
disruption in object discrimination behaviour by ischemic rats was a result of ischemia-
induced hippocampal damage. This is supported by the significant correlation between
the extent of CA1 cell loss and the magnitude of the impairment, indicated by the
discrimination ratio. However, the possibility remains that additional extra-
hippocampal neuronal damage may have occurred as a result of ischemia, but remained
undetected. Therefore the precise extent of the underlying pathology responsible for the
behavioural deficit cannot be defined with certainty.
It is clear that the one-trial object discrimination task used here is sensitive to ischemia-induced damage, and therefore may prove useful for testing the efficacy of potential anti-ischemic drugs at a behavioural level. However, its relation to other non-spatial tasks used to assess humans and monkeys remains unclear. As discussed above, the lack of discrimination between a novel object and an object seen previously by ischemic rats may reflect a disruption in novelty-preference as opposed to a memory deficit. In addition, normal discrimination between the novel and sample objects may not require the kind of memory (that is, declarative or explicit memory) that is thought to be affected in amnesic patients. Instead, the tendency of normal rats to explore a novel object more than an object to which they have been exposed previously may reflect non-declarative processes, such as habituation to the sample object. Consequently, this task may not be ideal for a rat model of ischemia-induced amnesia. Instead, it is necessary to use tasks that are more directly comparable to tasks used to tests human amnesics and monkeys.

Experiment 3.2: Delayed Nonmatching to Sample in Naive Rats

INTRODUCTION

Recently, a task analogous to the monkey DNMS task has been developed for rats (Mumby, Pinel, & Wood, 1990). This task has been designed to mimic the monkey DNMS task along several dimensions including the response requirements and the test stimuli used. The performance of normal rats on DNMS task is comparable to that reported for monkeys in terms of both the rate at which they learn the non-matching rule, and their choice accuracy at several retention delays (Mumby et al.,
1990). In addition, it appears that DNMS performance involves similar memory abilities in humans, monkeys and rats; the addition of delays and/or distraction during the delay affects the performance of rats, monkeys and humans in a similar fashion. The sensitivity of DNMS performance by rats to distraction suggests that their performance involves explicit memory, as distraction has been shown to disrupt the performance of human subjects on explicit memory tasks, but not on implicit memory tasks (Graf & Schacter, 1987).

The purpose of the present experiment was to assess the effect of ischemia-induced brain damage on the acquisition of DNMS at a short (4-s) retention delay and on subsequent performance at various delays up to 5-min by rats that received no presurgery training on DNMS. The relationship between the degree and distribution of ischemia-induced cell loss and the behavioral deficits was also assessed.

**METHOD**

**Subjects**

The subjects were 12 male Wistar rats (Charles River, Quebec) that weighed 300-325g at the beginning of the experiment. They had not been used in any previous experiments. However, the anatomical data from 6 of the rats (3 controls and 3 ischemics) were used in the analyses in Chapter 2. They were housed individually under a 12/12 hour dark/light cycle with unrestricted access to water. During behavioural testing they were maintained on a restricted feeding schedule; they were fed once per day after the test session, and their body weights were reduced to 85% of
their free-feeding body weights and maintained at this level, allowing for normal weight gain.

**Apparatus**

The testing apparatus has been described in detail previously (Mumby et al., 1990). It consists of a straight runway with identical goal areas at each end that are separated from a central starting area by two guillotine doors. Each goal area contains two recessed food wells that are separated by a short dividing wall. Food pellets (45 mg Bio-Serv Inc., Frenchtown, N.J.) can be delivered to the food wells manually through silicone tubing connected to funnels mounted on the outside of the apparatus. The test stimuli were a collection of over 350 "junk" objects with distinctive shapes, colours, and textures. All were large enough to cover the food wells but small enough to be displaced easily by the rats.

**Surgery**

Acute transient forebrain ischemia was induced in 8 rats according to the procedures outlined in Chapter 2. Four sham-ischemia control rats underwent the same procedures with the exception of hemorrhaging and carotid artery occlusion. Postsurgical care of all animals was conducted as in Chapter 2.

**Neuropathological Assessment**

After behavioral testing, rats were sacrificed and their brains were prepared for histological analysis according to the procedures described in Chapter 2. Cell counts
and calculations of cell loss were performed for the hippocampus and extrahippocampal structures as described in Chapter 2.

**Behavioural Procedures**

The behavioural testing procedures were similar to those reported previously (Mumby et al., 1990). All testing occurred during the light phase of the light/dark cycle, between 14 and 21 h after the rat’s most recent meal. Each rat was tested no more than once per day and no less than five times per week. Before training, the 12 rats were food deprived to 85% of their free-feeding body weights and maintained at this level during behavioural testing. Prior to ischemia induction, all rats were habituated to the apparatus and trained on a simple object-discrimination task. Following surgery, the rats progressed through three phases of training and testing: first they were retested on the same object-discrimination task learned before surgery; second they were trained on DNMS at a 4-s retention delay; and finally they were tested on DNMS at several longer retention delays.

**Preoperative training.**

The habituation phase consisted of five 20-min sessions. During the first three session the rats were shaped to run back and forth between the two goal areas. This was achieved by baiting one food well at a time at alternate ends of the apparatus and leaving the doors open, so that the rats learned to run from end to end to find food. On sessions 4 and 5, the operation of the guillotine doors was introduced, and the experimenter shaped the rats to approach the doors by baiting a food well on the far side of a closed door and raising the door when the rat approached it.
Following habituation each rat was trained on an object-discrimination task. Each rat received 25 trials daily. Two test objects were used for each rat during the object-discrimination training; one was randomly designated as S+ (reward) and the other as S- (no reward). At the beginning of each session, one door was closed and the S+ and S- were placed over the two food wells behind the door. The rat was placed in the centre of the apparatus, and when it approached the closed door the door was opened allowing access to the S+ and S-. If the rat displaced S+ from over its food well, a food pellet was delivered to that food well; if it displaced S-, no reward was delivered. After the rat had displaced one of the objects, the far door was lowered, and the S+ and S- were positioned over the two food wells behind it in preparation for the next trial. The positions (left versus right) of S+ and S- varied across trials in an irregular but balanced pattern. During the first session of 25 trials, the rats were allowed to correct themselves—if a rat displaced S-, it was allowed subsequently to displace S+ and was rewarded for this. However, on subsequent sessions no correction was allowed; both objects were removed as soon as either S+ or S- had been displaced. Daily object-discrimination sessions continued until a criterion of at least 21 correct choices out of 25 on two consecutive sessions was achieved. Following the object-discrimination phase, the rats were given unrestricted access to lab chow and allowed to return to a normal undeprived body weight before surgery.

Postoperative training and testing.

After 21 days of postsurgery recovery, the surviving ischemic rats and sham-surgery control rats were replaced on the restricted feeding schedule. Postoperative testing began 14 days later (5 weeks after surgery). Rats were first retested on the
object-discrimination task. Object-discrimination sessions were conducted exactly as before, with the same $S^+$ and $S^-$, until the criterion of 21 correct out of 25 on two consecutive sessions had been achieved once again.

Next, the rats were trained on DNMS. The test objects were divided into seven sets of 50, and different sets were used on successive sessions. For each of the 25 trials within a session, a different pair of objects was used. Each rat started each session in the central area with both doors closed. Two objects were picked from the pool; one was designated as the sample, and the other as the novel object. The sample and novel objects were placed over randomly designated food wells, one at each end. One of the doors was then opened to allow access to the sample object, which the rat displaced to obtain a food pellet. The sample object was then removed and placed over the vacant food well at the other end of the apparatus. After a 4-s retention delay, the second door was opened, and the rat was allowed to displace one of the two objects. The rat was rewarded if it chose the novel object but not if it chose the sample object. If a rat merely touched the object, but did not displace it, it was not considered to have made a choice. As in the preoperative object discrimination training, correction was allowed for the first session of 25 trials but not thereafter. After a rat had made a choice, the objects were removed, and both doors were closed once the rat had returned to the start area. New sample and novel objects were then chosen and positioned for the next trial. Rats were not handled during DNMS sessions. Each rat received 25 trials per session with a 4-s retention delay until they reached the criterion of at least 21 correct out of 25 on two consecutive sessions. The retention delay was defined as the period between the rat displacing the sample object and the opening of the second door to allow access to the sample and novel objects.
After reaching criterion at the 4-s retention delay, each rat’s performance at increasing retention delays was assessed. Rats were tested for eight sessions of 25 trials at each of the following retention delays: 4, 15, 30, 60, 120, and 300 s.

**RESULTS**

**Neuropathology**

Two animals in the ischemic group died within 72 hours of surgery; this left 6 ischemic rats and 4 sham-ischemia control rats. The results of the cell counts in the pyramidal cell layer of the CA1, CA2-3, and CA4 hippocampal cell fields, in the dentate gyrus granule cell layer, in the hilus of the dentate gyrus, in the striatum, in the mammillary nuclei, and in the entorhinal and perirhinal cortices are summarized in Figure 9. A repeated measures ANOVA on the averaged cell counts was performed, using group and brain area as independent variables. The data did not satisfy the assumption of sphericity. Accordingly, degrees of freedom were adjusted using the Greenhouse-Geisser Epsilon. The overall difference between the two groups was not statistically significant ($F=2.83, p>0.05$); however, there was a significant group x brain area interaction ($F=7.93, p<0.01$). Post hoc analyses (Tukey) revealed that ischemic rats had significantly fewer CA1 pyramidal neurons ($p<0.01$) and dentate hilar neurons ($p<0.05$) than control rats, but that they suffered no significant cell loss in any of the other brain areas in which cells were quantified.

Averaged across the entire septotemporal extent of the hippocampus, the degree of CA1 pyramidal cell loss in ischemic rats ranged from 38.8% for the least affected animal to 89.9% for the most affected. In order to analyze the pattern of CA1 cell loss
Figure 9. Histograms of the mean cell counts for sham-ischemia control rats (solid bars) and ischemic rats (hatched bars) expressed as a percentage of the mean cell counts for sham-ischemia control rats in each of the following areas: pyramidal neurons in CA1, CA2-3, and CA4, dentate granule cells (DG-G), dentate hilar neurons (DG-H), striatal neurons (STRIAT), entorhinal cortex neurons (ENT), perirhinal cortex neurons (PERI), and mammillary nuclei neurons (MAMM). Vertical bars show standard errors, ** denotes significance at $p < 0.01$; * denotes significance at $p < 0.05$. 
along the septotemporal extent of the hippocampus, a repeated measures ANOVA of the CA1 cell counts across the six hippocampal levels for the two groups was performed. This analysis revealed an overall significant difference between the two groups ($F=12.51$, $df=1$, $p<0.01$), a significant effect of level ($F=4.68$, $df=5$, $p<0.01$) and a significant group x level interaction ($F=6.75$, $df=5$, $p<0.01$). Post hoc analyses (Tukey) revealed that the ischemic rats had significantly fewer CA1 pyramidal neurons than controls at all levels except the most temporal level counted (level 6)—see Figure 10.

Inspection by light microscopy of areas of the brain that were not subjected to formal cell counts (e.g. various diencephalic nuclei, the amygdala and various cortical areas) revealed no observable damage to these structures.

**Behaviour**

Figure 11 summarizes the performance of ischemic and control rats on the object-discrimination task, before and after surgery. Before surgery, all rats learned the discrimination to the criterion within five sessions. When they were retested following surgery, all of the ischemic and sham operated control rats re-achieved the criterion within two sessions. A repeated measures ANOVA on the mean performance on the last two sessions before surgery and the two sessions after surgery revealed no significant main effect for groups or for time of testing (presurgery versus postsurgery), and no group x time of testing interaction; that is, there were no significant differences between the scores of the ischemic and control rats either before surgery or after surgery, nor were there any differences between the scores of either group on the last two preoperative days compared to the two postoperative days.
Figure 10. Density and distribution of ischemia-induced CA1 cell loss from the septal end (level 1) to the temporal end (level 6) of the hippocampal formation for the rats in Experiment 3.2. The percentage of CA1 cell loss at each level, and the mean for the 6 levels combined (X) are shown as horizontal bars (mean ± SE). The corresponding septo-temporal levels are depicted on the right. ** denotes significant cell loss at p < 0.01; * denotes significant cell loss at p < 0.05. The drawings of the hippocampus are adapted from Auer, Jensen, & Whishaw (1989).
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The figure illustrates the percentage of CA1 cell loss across different septo-temporal levels (1 to 6). Each level is represented by a bar graph, with the x-axis showing the percentage of cell loss ranging from 0 to 100. Significant differences are indicated by asterisks: * for a p-value less than 0.05 and ** for a p-value less than 0.01. The right side of the image shows a detailed illustration of the brain structures corresponding to these levels.
Figure 11. Histograms of mean percent correct over two sessions of 25 trials on an object-discrimination task before surgery (pre) and after surgery (post) for control and ischemic rats. Vertical bars show standard errors.
OBJECT-DISCRIMINATION

% Correct

Pre  Post

Control  Ischemic

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Ischemic rats were significantly impaired on acquisition of DNMS (see Figure 12); they took longer to reach criterion at the 4-s retention delay than control rats (mean number of trials to criterion for control rats = 350, and for ischemic rats = 725; $df=8$, $t=7.13$, $p<0.05$). Two of the ischemic rats failed to reach criterion after 40 sessions (1000 trials); on several occasions they achieved a score of $>21$ in a session, but never on two consecutive sessions. After 40 sessions (1000 trials) at the 4-s retention delay these two rats were tested at the other retention delays as described in the Methods section. However the behavioural data from these two rats were excluded in the subsequent statistical analyses of performance across each of the retention delays.

Figure 13 illustrates the performance of ischemic rats ($n=4$) and control rats ($n=4$) across several retention delays. A repeated measures ANOVA revealed a significant difference between the two groups ($F=17.62$, $df=1$, $p<0.01$) and a significant effect of retention delay ($F=17.41$, $df=5$, $p<0.01$). The group x retention delay interaction was not statistically significant ($F=1.48$, $df=5$, $p>0.05$). Therefore, following ischemia rats were impaired at each of the delays.

In order to assess the relation between the neuropathological changes and the performance deficits induced by transient forebrain ischemia, correlations between the mean number of cells per 125 um, averaged across all six levels of the hippocampus, and both trials to criterion and percent correct at each of the retention intervals were calculated. The two rats that failed to reach the learning criterion were included in these analyses. There was a significant negative correlation between the number of CA1 cells remaining and acquisition of DNMS as shown in Figure 14a ($r=-0.8023$, $df=9$, $p<0.01$); rats with the fewest CA1 cells remaining took the longest to reach criterion at the 4-s retention interval. There was also a significant correlation between
Figure 12. Histograms of mean trials to criterion on delayed nonmatching to sample (DNMS) by control and ischemic rats that received no presurgery training. Vertical bars show standard errors.
Figure 13. Mean percent correct on DNMS with increasing retention delays in control and ischemic rats that received no presurgery training. Vertical bars show standard errors.
Figure 14. Number of viable CA1 pyramidal neurons for individual rats plotted against (A) trials to criterion on DNMS, and (B) performance on DNMS with a 30-s retention delay in sham ischemia control rats (solid circles) and ischemic rats (open circles) that received no presurgery training. The lines show the linear regression between the two variables.
A

TRIALS TO CRITERION

Trials

0 1000
800
600
400
200

0 10 20 30 40 50
CA1 Cells / 125 um

r = 0.8023, p<0.01

B

30-s DELAY

% Correct

0 100
90
80
70
60
50

0 10 20 30 40 50
CA1 Cells / 125 um

(r = 0.9338, p<0.01)
the number of CA1 cells remaining and performance at the 15-s, 30-s (shown in Figure 14b), 60-s and 120-s retention delays (all $p < 0.01$). The correlation was not significant at the 4-s and 300-s retention delays.

**DISCUSSION**

Following transient forebrain ischemia, the experimental rats in Experiment 2.2a were significantly impaired on DNMS. Ischemic rats took longer to acquire the nonmatching rule at a 4-s retention delay and their choice accuracy at retention delays of 15 s, 30 s, 60 s, 120 s, and 300 s was significantly worse than that of sham-ischemia controls. In contrast, ischemic rats displayed no impairments on the object-discrimination task, which they had learned before surgery, indicating that they were still capable of discriminating between the objects and responding to them. It is therefore unlikely that their impairment on DNMS reflected compromised perceptual or motor abilities.

The pattern of damage produced by transient forebrain ischemia was similar to that found in Chapter 2; ischemia produced damage within the hippocampus, in the absence of detectable cell loss in other structures. The primary lesion involved extensive loss of CA1 pyramidal neurons throughout the hippocampus, except at its most temporal extent. However, in contrast to the pattern of cell loss observed in Experiment 1, there was moderate loss of neurons in the hilus of the dentate gyrus. This is consistent with the known vulnerability of somatostatin-containing neurons in the hilus of the dentate gyrus (Schmidt-Kastner & Freund, 1991), and suggests that this model also produces hilar neuron loss. It is possible that hilar loss also occurred in Experiment 1, but remained undetected.
There was no significant loss of pyramidal cells in other cell fields in the hippocampus; of dentate granule cells; or of cells in the striatum, the entorhinal cortex, the perirhinal cortex or the mammillary nuclei.

Given the neuropathological profile of rats following transient forebrain ischemia, it is likely that the deficits on DNMS are a result of ischemia-induced hippocampal damage (although other possibilities are discussed later). This conclusion is supported by the significant correlations between the degree of CA1 cell loss and the magnitude of DNMS deficits; the rats with the fewest viable CA1 cells displayed the most profound impairment, in terms of both their initial learning of DNMS and their performance at delays longer than 4 s. The absence of a significant correlation at the longest delay, 300 s, may have been due to a floor effect--the performance of several rats was not substantially above chance at the 300-s delay.

**Experiment 3.3: Reacquisition and Performance of DNMS by Rats that were Trained Prior to Ischemia.**

**INTRODUCTION**

Experiment 3.3 examined the postsurgical performance of DNMS by ischemic and control rats that had extensive DNMS training prior to surgery. Experiments on nonhuman primates have found that hippocampal lesions affect the DNMS performance of monkeys that have received training before surgery less than that of monkeys that have received no training before surgery (e.g., Zola-Morgan & Squire, 1986). Similarly, ischemia has been shown to produce less severe deficits in radial-arm-maze performance in rats that have had presurgery training (Davis & Volpe, 1990). The
The purpose of this experiment was to assess the DNMS performance of ischemic rats that received presurgery DNMS training.

**METHOD**

**Subjects and Apparatus**

The subjects were 20 male Wistar rats. The housing and feeding conditions were the same as those in Experiment 3.2. The apparatus was the same as that used in Experiment 3.2.

**Surgery and Neuropathological Assessment**

All surgical, histological and quantitative procedures were the same as in Experiment 3.2.

**Behavioural Procedures**

At the beginning of Experiment 3.3, the rats were food deprived to 85% of their free-feeding body weights and maintained at this level throughout behavioural testing. Each rat was habituated to the apparatus, and then it progressed through three phases of presurgery training and testing: (1) acquisition of the object-discrimination task (as in Experiment 3.2), (2) acquisition of DNMS, and (3) determination of its presurgery retention function. The ability of the rats to reacquire DNMS at the 4-s retention delay was assessed 4 weeks after surgery, and then their postsurgery retention functions were determined.
Presurgery training

Habituation and object-discrimination training were conducted as in Experiment 3.2. After rats had reached criterion on the object-discrimination task, DNMS training commenced. Rats received 20 trials per day at a retention delay of 4 s, until they reached the criterion of at least 85% (17/20) correct on two consecutive days. Then, the retention delay was increased to 15, 30, 60, 120 and finally to 300 s. The delay was increased each time that a rat reattained the criterion (85% on two consecutive sessions) or completed eight sessions at a particular retention delay without achieving the criterion. In the final phase of presurgery testing, each rat's retention function was assessed. Rats received 10 mixed-delays sessions, each consisting of 24 trials. On these sessions, four trials were conducted at delays of 4, 15, 30, 60, 120, and 300 s.

Postsurgery testing

After 21 days of postsurgery recovery, the rats were replaced on the restricted feeding schedule. Beginning 14 days later (5 weeks after surgery), the rats were tested on the DNMS task at a 4-s delay until they reattained criterion (at least 85% correct on two consecutive sessions) or completed 50 sessions (1000 trials) without achieving criterion. Then, the postsurgery retention function of each rat was assessed. The rats were given 10 mixed-delay sessions following the same procedures that were used prior to surgery.
RESULTS

Neuropathology

8 animals in the ischemia group died within 72 hours of surgery; this left 6 ischemic rats and 6 sham-ischemia control rats. A repeated measures ANOVA was performed on the averaged cell counts for the various brain areas, using group and brain area as independent variables. The degrees of freedom were adjusted using the Greenhouse-Geisser Epsilon, as the data did not satisfy the assumption of sphericity. The overall difference between the two groups was not statistically significant ($F=0.63$, $p>0.05$). There was a significant group x brain area interaction ($F=19.84; p<0.01$). Post hoc analyses (Tukey) showed that the ischemic rats had significantly fewer CA1 pyramidal neurons than control rats ($p<0.01$), but that there were no significant differences between the two groups in the cell counts in any other brain areas in which cells were quantified.

The degree of CA1 pyramidal cell loss in the ischemic rats ranged from 46.7% for the least affected animal to 89.9% for the most affected. A repeated measures ANOVA of the CA1 cell counts across the six septo-temporal levels of the hippocampus for the two groups revealed an overall significant difference between the two groups ($F=61.91$, $df=1$, $p<0.01$), a significant effect of level ($F=3.26$, $df=5$, $p<0.05$) and a significant group x level interaction ($F=5.12$, $df=5$, $p<0.01$). Post hoc analyses (Tukey) showed that ischemic rats had significantly fewer CA1 pyramidal neurons than controls at each of the six levels ($ps<0.01$).

As in Experiment 3.2, inspection of brain areas that were not subjected to formal cell counts revealed no observable damage.
Behaviour

The ischemic rats were significantly impaired on reacquisition of DNMS following surgery, although all rats reached the learning criterion within 1000 trials (Figure 15). A repeated measures ANOVA on the number of trials to criterion with groups as the between factor and time-of-testing (preoperative versus postoperative) as the repeated-measures factor revealed a significant main effect for group ($F=14.07$, $df=1, p<0.01$) and a significant group x time-of-testing interaction ($F=6.74$, $df=1$, $p<0.05$). The main effect for time-of-testing was not significant. Post hoc analyses (Tukey) revealed that there were no differences between the two groups before surgery, but following surgery, the ischemic rats took significantly more trials to achieve criterion than controls ($p<0.01$). Control rats required significantly fewer trials to achieve criterion following surgery than they did prior to surgery (mean number of trials to criterion before surgery = 347, and after surgery = 50; $df=5, t=6.04, p<0.01$). In contrast, the number of trials that ischemic rats required to reattain criterion after surgery was not significantly different from the number they required before surgery (mean number of trials to criterion before surgery = 450, and after surgery = 580; $df=5, t=0.83, p>0.05$ (paired t-tests corrected for multiple comparisons by the Bonferroni procedure).

The mean retention functions of ischemic and control rats before and after surgery are depicted in Figure 16. A repeated measures ANOVA with one between subjects factor (group) and two repeated measures factors (time-of-testing and retention delay) revealed a significant difference between the two groups ($F=7.17$, $df=1$, $p<0.05$), a significant main effect of time of testing ($F=10.06$, $df=1$, $p<0.05$), and a significant main effect of delay ($F=20.87$, $df=5$, $p<0.01$). The group x time-of-
Figure 15. Histograms of mean trials to criterion on DNMS before (pre) and after (post) surgery by rats that received presurgery training. Vertical bars show standard errors.
Figure 16. Presurgery (solid lines) and postsurgery (dashed lines) retention functions determined on the mixed-delay DNMS sessions by rats that received presurgery training. Vertical bars show standard errors.
testing interaction was the only significant interaction ($F=5.53$, $df=1$, $p<0.05$). Post hoc analyses showed that there were no significant differences between the two groups prior to surgery ($F=0.31$, $df=1$, $p>0.05$) but that the ischemic rats performed significantly worse than the controls after surgery across all delays (4 s, 15 s, 30 s, 60 s, 120 s, and 300 s) ($F=9.22$, $df=1$, $p<0.05$).

In order to determine whether there was a relationship between the extent of CA1 pyramidal cell loss and the DNMS performance deficits similar to that seen in Experiment 2.2a, correlations between the mean number of cells per 125 um, averaged across all six levels of the hippocampus, and both trials to criterion and percent correct at each of the retention delays were calculated. There was a significant negative correlation between the number of CA1 cells and reacquisition of DNMS following surgery as shown in Figure 17A ($r=-0.7522$, $df=11$, $p<0.01$). There was also a significant correlation between the number of CA1 cells and performance at the 4-s, 15-s, and 30-s (shown in Figure 17B) retention delays (all $ps<0.05$). However, the correlations at the 60-s, 120-s, and 300-s delays were not statistically significant.

**DISCUSSION**

The results of Experiment 3.3 show that transient forebrain ischemia impairs the DNMS performance of rats that have had extensive experience with the DNMS task prior to ischemia. Following surgery, the ischemic rats took significantly longer to reattain the criterion at the 4-s delay than did the control rats. Furthermore, the ischemic rats showed no evidence of preserved memory for the DNMS rule learned prior to ischemia; the mean number of trials needed to reach criterion following ischemia was not significantly different from that required to reach criterion during
Figure 17. Number of viable CA1 pyramidal neurons for individual rats plotted against A postsurgery trials to criterion on DNMS, and B postsurgery performance on DNMS with a 30-s retention delay in sham ischemia control rats (solid circles) and ischemic rats (open circles) that received presurgery training. The lines show the linear regression between the two variables.
initial training. In contrast, control rats showed significant memory for their presurgery training; they took significantly fewer trials to reach criterion after sham surgery than during initial training. Ischemic rats displayed significant postsurgical DNMS impairment relative to the control rats at all retention intervals. The degree of impairment was comparable at all delays (see Figure 16); the ischemic rats were significantly impaired even at the shortest delay. The degree of DNMS impairment was similar to that observed in Experiment 3.2. Therefore, extensive presurgery training did not attenuate the DNMS deficits of the ischemic rats.

The fact that ischemic rats required a similar number of trials to reattain criterion after surgery as they required to reach criterion originally suggests that ischemia produced a retrograde amnesia for the DNMS task. However, the ischemic rats were clearly able to make use of some of the information that they had acquired prior to ischemia. For example, they displaced objects and searched for food in the food wells efficiently on the first trial following ischemia, suggesting that they were able to remember some procedural aspects of the task.

The pattern and severity of ischemia-induced cell loss in these rats was similar to that found in Chapter 2 and in Experiment 3.2, with the exception of a lack of hilar neuron loss; observable damage was limited to CA1 pyramidal neurons. It therefore appears that ischemia-induced hippocampal cell loss results in comparable DNMS impairments in rats with or without extensive presurgery training. This finding contrasts with those from studies utilizing spatial memory tasks to assess ischemia-induced memory deficits which report a strong relationship between the amount of presurgery training and postsurgery performance on spatial radial maze tasks by ischemic rats (e.g. Davis & Volpe, 1990). The significance of these differences with
respect to clinical cases of ischemia-induced amnesia is not clear, although it may be more useful to compare the effects of presurgery training on performance of one type of task, rather than to generalize across spatial and nonspatial tasks.

As in Experiment 3.2, there were significant correlations between the degree of CA1 pyramidal cell loss and DNMS performance; rats with the fewest CA1 neurons were slowest to relearn DNMS, and were the most impaired at delays up to 30 s. These findings are consistent with the idea that ischemia-induced hippocampal damage mediates the DNMS performance deficits. However, the absence of significant correlations between CA1 cells and DNMS performance at the longer delays (60 s, 120 s, and 300 s), despite the finding that ischemic rats perform significantly worse than control rats at these delays, leaves open the possibility that factors other than the degree of CA1 cell loss contribute to performance deficits at long delays.

**DISCUSSION OF EXPERIMENTS 3.2 AND 3.3**

There were two main findings in Experiments 3.2 and 3.3. First, transient forebrain ischemia resulted in impaired performance on DNMS both in rats with no preoperative training and in rats with extensive preoperative training. Second, there was a significant correlation between the degree of cell loss within the CA1 pyramidal cell field of the hippocampus, and the magnitude of the DNMS impairment.

Ischemic rats required more trials to learn the DNMS task with a 4-s retention interval, and their subsequent performance at retention intervals up to 5 min was significantly impaired, relative to sham-ischemia controls. Ischemic rats that had received extensive training and experience on DNMS prior to surgery showed no savings when tested after surgery. Following ischemia, they required as many trials to
reattain criterion at the 4-s delay as they had needed to learn the task initially. However, they were able to learn the task following ischemia slightly more quickly than the ischemic rats in Experiment 3.2, suggesting that there might have been some small savings afforded by prior training. Preoperative training did not affect the retention functions of ischemic rats; ischemic rats that had training before surgery were as impaired as those that had no prior training on DNMS. Furthermore, ischemia resulted in profound deficits even at the shortest retention delays, indicating that ischemic damage disrupted processes involved in memory for recent events.

The impaired performance of ischemic rats cannot be attributed to an inability to see or to move the objects, as performance on the two choice discrimination task, which required the same sensory and motor abilities as the nonmatching task, was the same for ischemics and controls. This conclusion is supported by the observation that the ischemic rats were able to learn the DNMS task, albeit at a significantly slower rate. However, it is possible that the behavioural impairment was not due solely to a recognition memory deficit, but reflected disruption of attentional processes, increased susceptibility to distraction, or the use of a less efficient strategy for solving the task.

These findings complement those of the studies that have shown impaired performance on spatial memory tasks following ischemia in rats (Auer et al., 1989; Davis & Volpe, 1990; Hagan & Beaughard, 1990; Jaspers et al., 1990; Kiyota et al., 1991; Ordy et al., 1988), by extending the description of impairments following ischemia to a nonspatial memory task. The DNMS deficits that ischemic rats display are similar to deficits seen on spatial memory tasks--working memory aspects of the task are affected to a greater extent than reference memory aspects. The DNMS task is primarily a working memory task, as correct performance requires that rats remember
information that varies from trial to trial. However, there are also reference memory components including memory for the non-matching rule and for the other invariate aspects of the task. The finding that ischemic rats can learn the nonmatching rule, as indicated by their ability to reach criterion at the shortest retention delay, suggests that they can perform some reference memory components of the task. It is their performance on the working memory aspects that is most severely impaired.

This demonstration of DNMS deficits in ischemic rats permits the direct comparison of ischemia-induced memory deficits between rats, primates, and humans. While it is not possible to determine whether the performance of rats (or monkeys) is based on declarative (or implicit) memory due to the dependence of these definitions on verbal ability and/or consciousness, similar deficits in rats, monkeys and humans on the same task allow comparisons to be made between the species in terms of the nature of the memory deficits, and the ischemia-induced pathological changes underlying these deficits.

As found in Chapter 2, a 20-min period of ischemia produced by a combination of bilateral carotid occlusion and hypotension resulted in brain damage that appeared to be limited to the hippocampus. CA1 pyramidal neurons were destroyed in all rats, and there was also loss of some dentate hilar neurons. In general, there was greater CA1 cell loss in the rostral hippocampus than in its more caudal portions, as has been described previously (Auer et al., 1989; Ashton et al., 1989; Smith et al., 1984b).

Although no detectable extrahippocampal cell loss was observed, it is possible that subtle ischemia-induced neuronal changes may have contributed to the behavioural impairments. For example, loss of a small specific subset of neurons within one of the areas may not have been detected using cell counts that did not distinguish between
neuronal types. In addition, ischemia-induced functional changes in the physiology of neurons would not have been detected.

**Conclusion**

These experiments have extended the reports of recognition-memory deficits following ischemic brain damage in humans and monkeys to rats. The demonstration of ischemia-induced deficits by rats on a DNMS task that is analogous to the monkey DNMS task allows direct comparisons between rats, monkeys and humans in terms of both the neurobehavioural consequences of ischemia, and the neuropathological basis of the memory impairments. The correspondence between species will be discussed in the General Discussion.

This model may also prove to be a useful tool in assessing the ability of pharmacological agents to prevent ischemia-induced amnesia, especially given the relationship between the degree of neuronal damage and the magnitude of the impairments.
CHAPTER 4: EFFECTS OF 7-CHLOROKYNURENIC ACID ON THE NEUROPATHOLOGICAL AND BEHAVIOURAL CONSEQUENCES OF TRANSIENT FOREBRAIN ISCHEMIA

The experiments in Chapter 3 have provided the basis of a rat model of ischemia-induced amnesia that can be compared easily to ischemia-induced amnesia in monkeys and humans. One of the aims in the development of such a model was to provide a behavioural index that could be used to measure the protective properties of potential therapeutic drugs in transient forebrain ischemia. As discussed in the General Introduction, several different pharmacological strategies have been employed in the attempt to prevent ischemia-induced cell loss. However, only a few studies investigating the protective abilities of drugs have assessed efficacy at a behavioural level (e.g. Grotta et al., 1988; Malgouris et al., 1989; Rod, Whishaw & Auer, 1990 ). In these studies, the relationship between histological and behavioural indices of protection have not been consistent: in one study the glutamate release blocker riluzole prevented both CA1 neuronal necrosis and passive avoidance impairments produced by ischemia in gerbils (Malgouris et al., 1989 ), suggesting that histological protection may be related to prevention of memory deficits. Rod et al. (1990) reported that MK801 significantly decreased the cell loss produced by transient forebrain ischemia in rats, but caused a less robust improvement in behavioural performance on a learning set task. As in the gerbil study there was a significant correlation between neuronal necrosis and neurobehavioural outcome. In contrast, Grotta et al. (1988 ) found that an L-type calcium channel blocker, nicardipine, prevented spatial working memory errors in rats without significantly decreasing ischemia-induced hippocampal damage. Taken together, these findings indicate that neural protection is not necessarily accompanied
by the amelioration of ischemia-induced deficits in learning and memory. As deficits in brain function, not histology, are the impairments of primary importance to survivors of ischemic episodes, it is clearly important to supplement anatomical evaluation of protection with behavioural evaluation.

Accordingly, the experiments in this chapter investigate the ability of a potential neuroprotectant to prevent both ischemia-induced histological changes, and ischemia-induced memory deficits.

**Experiment 4.1: Histological Analysis of the Neuroprotective Effects of 7-Chlorokynurenic Acid**

*INTRODUCTION*

As discussed in the General Introduction, a prominent theory concerning the mechanism of ischemia-induced cell loss is that it is mediated in part by the excitotoxic actions of excitatory amino acids (Rothman & Olney, 1986). In particular, excessive activation of both non-NMDA and NMDA glutamate receptors during ischemia is thought to mediate calcium influx, which in turn triggers various calcium-mediated processes culminating eventually in cell death (Choi, 1988b). One of the most promising approaches to the prevention of ischemia-induced cell death is the use of drugs that interfere with glutamatergic activity; both glutamate release blockers and glutamate receptor blockers have been shown to decrease the damage produced by transient forebrain ischemia in several animal models. These drugs appear to be most effective if administered before or during the period of ischemia. However, as discussed earlier, there are several negative features of the glutamate receptor
antagonists that may overshadow their therapeutic potential. Noncompetitive NMDA antagonists are effective only at doses that produce behavioural disturbances (Willetts et al., 1990), and both competitive and non-competitive NMDA antagonists have been shown to cause neurotoxic side effects in retrospenial and posterior cingulate cortex (Olney et al., 1991). The utility of noncompetitive NMDA antagonists may also be limited by the sensitivity of channel blockade to extreme membrane depolarisation (Miller, 1987). The therapeutic potential of non-NMDA receptor antagonists may also be limited, as blockade of AMPA and QUIS receptors may result in drastic depression of synaptic activity throughout the CNS.

One approach that has received little attention is the use of antagonists at the strychnine-insensitive glycine binding site associated with the NMDA receptor. As discussed earlier, glycine has been shown to potentiate NMDA channel activation (Johnson & Ascher, 1987) and it has been suggested that glycine may be a co-agonist at the NMDA receptor-channel complex. Specifically, simultaneous binding of glycine and glutamate to their respective binding sites on the NMDA receptor-channel complex is thought to be required, with concomitant cell depolarization, for ion conductance through the channel (Dingeldine et al., 1990; Kleckner & Dingeldine, 1988; Lehmann et al., 1991; Lerma et al., 1990). Therefore, blockade of the glycine site would be expected to prevent influx of calcium via the NMDA receptor. This approach may result in neuroprotection while avoiding the negative side effects of competitive and non-competitive NMDA receptor antagonists.

Several recent findings suggest that glycine may play a role in the pathological consequences of transient cerebral ischemia. A study utilizing the in vivo microdialysis technique showed that there was a close correlation between the areas of the brain that
suffered cell loss and those in which extracellular glycine was elevated during ischemia and remained elevated following reperfusion. In contrast, changes in extracellular glutamate levels during ischemia and reperfusion were equivalent across both vulnerable and non-vulnerable brain areas (Globus, Busto, Martinez, Valdes, & Dietrich, 1990). Glycine has also been shown to potentiate NMDA-mediated toxicity \textit{in vitro} (Patel et al., 1990). These findings suggest that elevated extracellular levels of glycine during and following ischemia may contribute to or potentiate the damage mediated by calcium influx via the NMDA channel. Prevention of glycine binding might therefore be expected to attenuate the cell loss associated with ischemia.

To investigate this possibility we have assessed the protective effects of 7-Chlorokynurenic acid (7-ClK)—a potent competitive antagonist at the strychnine-insensitive glycine binding site (Kemp et al., 1988)—against CA1 pyramidal cell loss in a rat model of transient forebrain ischemia.

\textit{METHOD}

\textbf{Subjects}

The subjects were 49 Male Wistar rats (Charles River, Quebec) that weighed 300-350g at the time of surgery. They were housed in groups of 6 under a 12/12 h light/dark cycle with unrestricted access to food and water.

\textbf{Surgery}

29 male rats received transient forebrain ischemia, and a further 20 rats received sham ischemia, using the procedures described in Chapter 2. Following surgery, each
rat was placed in a plastic cage in a recovery room and monitored closely for three
days. As described in Chapter 2, the ambient illumination and noise levels were kept as
low as possible to minimise the occurrence of seizures, and rats were aspirated if they
appeared to have problems breathing. However, to avoid confounding drug interaction
effects, rats were not administered atropine sulphate or diazepam at any time.

Half of the rats in each group received bilateral intraventricular injections of 2
ul 500 uM 7-ClK (Tocris Neuramin) at an infusion rate of 1 ul/min immediately before
carotid occlusion. The remaining rats received bilateral intraventricular injections of
vehicle (2 ul phosphate-buffered saline over 2 min). There were therefore 4 groups of
animals: ischemia + drug (ID, n=15), sham ischemia + drug (SD, n=14), ischemia +
saline (IS, n=10), sham ischemia + saline (SS, n=10).

Neuropathological Assessment

The rats were allowed to survive for either 6 or 12 weeks at which time they
were re-anesthetised with sodium pentobarbital (65 mg/kg ip) and perfused
transcardially with 10% formalin in 0.05% phosphate buffer. The brains were removed
immediately and prepared for histological analysis following the procedures described
in Chapter 2. The placements of the injection cannulae through which drugs were
delivered during ischemia were determined by examining the brain slices for the tips of
the cannula tracts. Quantification of CA1 pyramidal cells at each of 6 levels distributed
evenly along the septo-temporal axis of the hippocampus was performed as described
earlier. These cell counts were averaged across the right and left hippocampi to
generate a mean cell count expressed as cells per 125 um at each of the 6 levels for
each rat. Based on the lack of evidence for ischemia-induced cell loss in any of the
other hippocampal cell fields (except for the loss of a subset of hilar neurons), or in any of the extrahippocampal structures examined previously, cells were not quantified in these areas. During histological evaluation the experimenter was blind with respect to the experimental treatment of the animals.

RESULTS

Several of the rats subjected to ischemia exhibited motor seizures within three days of surgery, and of these, 5 in the ID group, and 4 in the IS group subsequently died. This left 10 rats in each of the four groups. In each of these rats, the injection cannulae had penetrated the ventricles, indicating that the injections were intraventricular.

7-Chlorokynurenic acid significantly attenuated ischemia-induced CA1 pyramidal cell loss (Figures 18 and 19). Relative to the SS group, the ischemic rats treated with 7-ClK (ID) had only 22.7% CA1 cell loss averaged across all levels of the hippocampus, whereas the ischemic animals not treated with the drug (IS) suffered 53.8% cell loss. One-way analysis of variance of the cell counts averaged across the six levels revealed a significant difference among the four groups ($F=15.046$, $df=3$, $p<0.01$). Post hoc analyses (Tukey tests) showed that the IS group had significantly fewer viable CA1 neurons than each of the control groups ($p<0.01$) and the ID group ($p<0.05$). The ID group also had significantly fewer cells than both control groups ($p<0.05$), but significantly more than IS ($p<0.05$). There were no significant differences between SS and SD groups. To investigate whether the pattern of CA1 cell loss varied across the different levels, a repeated measures two-way analysis of variance was conducted with groups and levels as independent variables. There was a
Figure 18. Representative photomicrographs of the hippocampal formation (a, b and c) and of part of the CA1 cell field (d, e and f) at level 2, from representative rats in the sham-ischemia + saline (SS) group (a and d), the ischemia + saline (IS) group (b and e) and the ischemia + drug (ID) group (c and f). Arrows on the low power photomicrographs indicate the region of CA1 from which the high power photomicrographs were taken. Ischemic damage in the IS condition can be seen in b as a thinner cell layer, and in e as a substantial decrease in the number of viable pyramidal cell bodies.
Figure 19. Histograms showing the mean (+ SE) CA1 cell counts expressed as a percentage of the mean cell count for the sham-ischemia + saline group, for each of the groups (IS, ID, SS, and SD) at each of the six hippocampal levels, and the mean cell loss for the six levels combined (X) in Experiment 4.1. (IS = ischemia + saline; ID = ischemia + drug; SS = sham-ischemia + saline; SD = sham-ischemia + drug) ** denotes significance at $p < 0.01$; * denotes significance at $p < 0.05$. 
### Chapter 4

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**Legend:**
- □ ISCHEMIA + SALINE (IS)
- × ISCHEMIA + 7CK (ID)
- ■ SHAM + SALINE (SS)
- ○ SHAM + 7CK (SD)
significant difference among the 4 groups \((F=12.18, \ df=3, \ p<0.01)\), and a significant group x level interaction \((F=5.65, \ df=15, \ p<0.01)\), but there was no significant main effect for level. Post hoc analyses (Tukey) showed that IS rats had significantly fewer CA1 cells than SS and SD rats at all 6 levels \((p<0.01)\). ID rats had fewer cells than SS and SD at levels 1 and 2 \((p<0.01)\), and 3 and 4 \((p<0.05)\), but not at levels 5 and 6. Ischemic rats treated with 7-ClK (ID) had more cells than untreated ischemic rats in levels 2, 4, 5 and 6 \((p<0.05)\), but not in levels 1 and 3.

DISCUSSION

Pre-ischemia administration of 7-Chlorokynuenic acid significantly attenuated ischemia-induced CA1 pyramidal cell loss. The degree of protection offered by 7-ClK was greater at more temporal levels of the hippocampus, while the drug was less effective in preventing CA1 cell loss at the septal pole. Previous studies have shown that the septal hippocampus is more susceptible to ischemia than the temporal hippocampus (Smith et al., 1984b), and this was also found in earlier experiments in this thesis. These findings suggest that 7-ClK was more effective in an area that is generally more resistant to ischemia. However, this cannot be confirmed, as the degree of cell loss in untreated ischemic rats (IS) was not significantly different across the 6 levels in this experiment.

The finding that 7-ClK reduces the CA1 pyramidal cell loss produced by transient forebrain ischemia suggests that activation of the glycine site on the NMDA receptor complex contributes to ischemia-induced neuronal death \textit{in vivo}. This finding is also consistent with the excitotoxic hypothesis of ischemia-induced neuronal damage, as antagonism of the glycine binding site on the NMDA receptor would be expected to
decrease NMDA channel opening (Dingledine et al., 1990), thus attenuating calcium entry via the NMDA receptor during ischemia. The fact that 7-ClK imparts only partial protection is not surprising, given that it competes with glycine for binding and, as discussed above, levels of glycine during ischemia are relatively high. Furthermore, only one dose of the drug was given in the present study. These data confirm a preliminary report of protective effects of glycine site antagonists against ischemia in gerbils (Patel et al., 1989), and are consistent with previous reports of protective effects of 7-ClK against NMDA-mediated neurotoxicity both in neocortical cultures (McNamara & Dingledine, 1989; Patel et al., 1990) and in vivo (Foster, Donald, Willis, Tridgett, Kemp, & Priestley, 1990).

These data do not address the question of the contribution of elevated glycine levels to NMDA receptor mediated damage during ischemia. One issue yet to be resolved is whether resting levels of glycine in the synapse are sufficient to fulfill the binding requirement for NMDA channel activation, or whether this requirement is met only when glycine levels are increased transiently, for example as a result of stimulated release from neurons or glia. Most in vitro evidence supports the theory that the glycine site is saturated tonically (Ascher, 1990; Kemp et al., 1988). However, addition of glycine or d-serine (a potent glycine site agonist) has been shown to potentiate NMDA responses in vivo (Danysz, Wroblewski, Brooker & Costa, 1989; Salt, 1989; Sing, Oles, & Tricklebank, 1990; Wood, Emmett, Rao, Mick, Cler, & Iyengar, 1989), suggesting that resting levels of glycine in the synapse may not be high enough to saturate the site completely. If this is the case, it is likely that elevated glycine during ischemia can potentiate glutamate's actions at the NMDA receptor. In addition to its role as a co-agonist, glycine may reduce desensitization of the NMDA receptor (Lerma
et al., 1990; Mayer et al., 1989). Under conditions of prolonged elevation of extracellular glutamate concentrations, as is the situation during ischemia, one might predict desensitization of the NMDA receptor site. However, in the presence of high extracellular glycine levels this desensitization may be prevented. Administration of a competitive glycine-binding site antagonist would therefore decrease the ability of glycine to potentiate the NMDA receptor-mediated response.

While 7-ClK is one of the most selective antagonists for the glycine site on the NMDA receptor (Kemp et al., 1988), and does not seem to affect the affinity of the NMDA binding site for either agonists or antagonists, it is only 40-fold more selective for the glycine site on the NMDA receptor than for the kainate binding site (Dingledine et al., 1990). Given that the density of kainate receptor expression on CA1 pyramidal cells is extremely low (Cotman et al., 1989), it is unlikely that the protective effects of 7-ClK against cell loss in CA1 can be explained by prevention of kainate receptor-mediated excitotoxicity. However, blockade of kainate receptors could interfere with mossy fibre transmission to CA3, and this could affect subsequent CA3 to CA1 transmission. In addition, 7-ClK may be effective in attenuating neuronal damage during ischemia by virtue of its free-radical scavenging properties (Moroni, 1991), as free radical formation during ischemia is thought to contribute to neuronal damage (Siesjo, 1981). It is also possible that there were unknown side effects of 7-ClK, such as hypothermia, which may have contributed to its neuroprotective effect. This possibility cannot be evaluated on the basis of the present findings.

Regardless of the mechanism of action, the experimental results provide strong evidence for the therapeutic potential of 7-ClK and other drugs acting at the glycine binding site on the NMDA receptor, against ischemia-induced cell loss.
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Experiment 4.2: Behavioural Analysis of the Neuroprotective Effects of 7-Chlorokynurenic Acid

INTRODUCTION

The results of Experiment 4.1 indicate that pre-ischemic administration of 7-Chlorokynurenic acid significantly decreases ischemia-induced CA1 cell loss. However, as discussed above, it is important to demonstrate the efficacy of a potential anti-ischemic drug at the behavioural level, as well as at the anatomical level. Accordingly, this experiment was designed to determine whether the neuroprotective effects of 7-ClK are concomitant with an improvement in behavioural performance. We chose to use acquisition of the DNMS task as a measure of behavioural outcome for two reasons: First, in Experiment 3.2 we had demonstrated a high correlation between the rate of acquisition of DNMS and the amount of CA1 cell loss. Second, as discussed earlier, acquisition deficits on DNMS have also been demonstrated following ischemia in monkeys (Bachevalier & Mishkin, 1989) and humans (Squire et al., 1988), indicating that this may be a useful measure of ischemia-induced deficits across species. In order to restrict the amount of time required to collect the behavioural data, we did not assess the effects of 7-ClK on ischemia-induced DNMS impairments at longer retention delays.
METHODS

Subjects

The subjects were 29 Male Wistar rats (Charles River, Quebec) weighing 300-325g at the time of surgery. Before surgery rats were housed in groups under a 12/12 h light/dark cycle with continuous access to water.

Apparatus

The apparatus was the same as that used in Experiment 3.2.

Surgery

Transient forebrain ischemia was induced in 17 rats, according to the procedures described in Chapter 2. A further 12 rats were subjected to sham ischemia. During the three day recovery period all rats were treated as described for Experiment 4.1.

As in Experiment 4.1, half of the ischemia rats and half of the sham-ischemia control rats received bilateral intraventricular injections of 2 ul 500 uM 7-CIK immediately before carotid occlusion. This yielded four groups of animals: ischemia + drug (ID, n=8), sham ischemia + drug (SD, n=6), ischemia + saline (IS, n=9), and sham ischemia + saline (SS, n=6).

Neuropathological Assessment

After behavioural testing, 12 weeks after surgery, rats were sacrificed and their brains prepared for histological analysis as described above. Injection cannulae
placements were determined and CA1 pyramidal cells were quantified as in Experiment 4.1.

**Behaviour**

After 21 days of postsurgery recovery the surviving rats ($n=6$ in each of the 4 groups) were housed individually and placed on a restricted feeding schedule to lower their body weights to 85%. Behavioural testing began 14 days later (5 weeks after surgery). Each rat was habituated to the apparatus, and then it progressed through two phases of testing: (1) acquisition of an object-discrimination task, and (2) acquisition of DNMS. The procedures for habituation, object discrimination and acquisition of DNMS were the same as those described in the presurgery training and testing procedures of Experiment 3.3.

**RESULTS**

**Neuropathology**

Three rats is the IS group and two rats in the ID group died the day after surgery: this left six rats in each of the four groups. Upon investigation of the brains, it was observed that one rat in the ID group had sustained a large amount of damage in the rostral half of the brain; the ventricles were grossly enlarged, and the striatum, thalamus and hippocampus appeared misshapen. We surmised that this rat had suffered mechanical brain damage, perhaps related to the drug injection. As this pattern of damage was not representative of that produced in either of the two ischemia groups,
and was unlike anything we have observed previously, the histological and behavioural data from this rat were excluded from the subsequent analyses.

7-Chlorokynurenic acid significantly decreased ischemia-induced CA1 pyramidal cell loss. The ischemic rats treated with 7CIK (ID) suffered only 21.9 ± 7.2% cell loss compared to sham ischemia saline control rats (SS), whereas the ischemic rats that received saline (IS) suffered 57.0 ± 6.5% cell loss. One-way analysis of variance on the CA1 pyramidal cell counts averages across the six levels revealed a significant difference among the four groups ($F=23.24$, $df=3$, $p<0.01$). Post hoc analyses (Tukey tests) showed that the IS group had significantly fewer neurons than each of the control groups (SS and SD), and the ID group ($p<0.01$). The ID group had significantly fewer CA1 cells than the SS group ($p<0.05$), but not significantly fewer than the SD group. The two control groups (SS and SD) did not differ significantly.

To determine whether the pattern of cell loss varied across the septo-temporal extent of the hippocampus, a two-way repeated measures ANOVA was conducted, with groups as the between subjects variable, and hippocampal level as the repeated measures factor. The degrees of freedom were adjusted using the Greenhouse-Geisser Epsilon, as the data did not satisfy the sphericity assumption. There was a significant difference among the four groups ($F=23.24$, $p<0.01$), a significant effect of hippocampal level ($F=5.61$, $p<0.01$), and a significant group x level interaction ($F=4.37$, $p<0.01$). Post hoc analyses (Tukey) showed that IS rats had significantly fewer CA1 cells than SS and SD rats at each of the six levels ($p<0.01$). ID rats had fewer cells than SD rats at level 1, and less than SS rats at levels 2 and 3 ($p<0.05$). At levels 4, 5, and 6 the ID rats were not significantly different from either of the
control groups (SS and SD). Comparing the two ischemic groups, ID rats had more CA1 cells than IS rats at all six levels (levels 1, 2, 3, and 4, \( p < 0.05 \); levels 5 and 6, \( p < 0.01 \)). These data are illustrated in Figure 20.

**Behaviour**

Figure 21 illustrates the trials required to reach criterion on the object-discrimination task by rats in the four groups. All rats learned the discrimination within five sessions of 25 trials, and a one-way ANOVA on the number of trials required to reach criterion on the object-discrimination showed that there were no significant differences between the groups (\( F=0.26, \ df=3, \ p > 0.05 \)).

Figure 22 illustrates the mean trials required to achieve criterion on DNMS by rats in each of the four groups. The IS rats required a mean \( 586.7 \pm 67.9 \) trials to reach criterion, while ID rats required only a mean \( 396 \pm 41.2 \) trials. The SS and SD required \( 416.7 \pm 40.14 \) and \( 346.7 \pm 67.5 \) respectively. A Students t-test on the trials to criterion for the two control groups (SS and SD) showed that 7-Chlorokynurenic acid did not significantly affect the ability of sham-ischemia control rats to learn the DNMS task (pooled \( t=0.89, \ df=10, \ p > 0.05 \)). In addition, the cell counts for these two groups were not significantly different (pooled \( t=1.73, \ df=10, \ p > 0.05 \)). Accordingly, the behavioural data from the two control groups was combined for subsequent analysis. A one-way ANOVA on the trials to criterion for the three groups (combined controls, IS and ID) revealed a significant difference among the groups (\( F=5.15, \ df=2, \ p < 0.05 \)). Post hoc tests (Newman-Keuls) revealed that the IS group required significantly more trials to reach criterion than both the combined control group and the
Figure 20. Histograms showing the mean (+ SE) CA1 cell counts expressed as a percentage of the mean cell count for the sham-ischemia + saline group for each of the groups (IS, ID, SS and SD) at each of the six hippocampal levels, and the mean cell loss for the 6 levels combined (X), in Experiment 4.2. (IS = ischemia + saline; ID = ischemia + drug; SS = sham-ischemia + saline; SD = sham-ischemia + drug) ** denotes significance at $p < 0.01$; * denotes significance at $p < 0.05$. 
Figure 21. Histograms of mean trials to criterion on object-discrimination by sham-ischemia + saline (SS), sham-ischemia + drug (SD), ischemia + saline (IS) and ischemia + drug (ID) rats. Vertical bars show standard errors.
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OBJECT-DISCRIMINATION

Trials to criterion

SS  SD  IS  ID

120
100
80
60
40
20
0
Figure 22. Histograms of mean trials to criterion on delayed nonmatching to sample (DNMS) by sham-ischemia + saline (SS), sham-ischemia + drug (SD), ischemia + saline (IS) and ischemia + drug (ID) rats. Vertical bars show standard errors.
Trials to criterion

SS  SD  IS  ID

DNMS

Chapter 4
ID group ($ps < 0.05$). The difference between the ID group and the combined control group was not significant.

As in previous experiments, the relationship between the degree of CA1 cell loss and the trials required to reach criterion on DNMS was assessed. There was a significant negative correlation between the number of intact CA1 pyramidal neurons averaged across the six levels of the hippocampus, and the trials to criterion ($r=0.4337$, $df=22$, $p < 0.05$). Rats with the fewest CA1 cells tended to require more trials to learn the DNMS task—see Figure 23.

**DISCUSSION**

This experiment has replicated the histological findings of experiment 3.1. 7-Chlorokynurenic acid administered immediately prior to bilateral carotid occlusion significantly attenuated ischemia-induced CA1 pyramidal cell loss in rats. It has also provided preliminary evidence that the neuroprotective effect of 7-ClK was associated with a significant improvement in behavioural outcome; ischemic rats that received 7-ClK (ID) learned the DNMS task in significantly fewer trials than ischemic rats administered saline (IS), and their performance was not significantly worse than that of control rats. The results of experiment 3.2 indicated that trials to criterion on DNMS is one of the most sensitive indices of ischemia-induced disruption of learning in this model of ischemia. Therefore, the ability of 7-ClK to ameliorate the DNMS acquisition deficits of ischemic rats suggests that this drug may be capable of preventing ischemia-induced memory deficits. However, this cannot be confirmed without more extensive assessment of the behavioural outcome of 7-ClK in ischemic rats.
Figure 23. Number of viable CA1 pyramidal neurons for individual rats plotted against trials to criterion on DNMS for sham-ischemia + saline (SS, solid circles), sham-ischemia + drug (SD, solid squares), ischemia + saline (IS open circles) and ischemia + drug (ID, open squares) rats. The line shows the linear regression between the two variables.
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![Graph](image)

- **Trials to criterion** vs. **CA1 cells / 125 um**

- Correlation coefficient: $r = 0.4337; p < 0.05$
Since there was no significant difference between the drug-treated and saline-treated sham-ischemia rats, the results suggest that the amelioration of DNMS deficits by 7-ClK in ischemic rats was due to a reduction in ischemic brain damage in areas responsible for normal performance on DNMS, rather than to other, non-specific actions of the drug. Given the significant correlation between CA1 cells and acquisition of DNMS, the findings are consistent with the hypothesis that CA1 cell sparing by 7-ClK accounts for the improved behavioural outcome. However, as discussed earlier, pathological changes outside the hippocampus may also contribute to recognition memory deficits. Therefore, it is possible that the improvement in DNMS performance by ID rats may be due to neuroprotection outside the hippocampus, or to a combination of hippocampal and extrahippocampal cell sparing.

Regardless of the neuropathological basis of the memory impairments following ischemia, these results provide compelling evidence that 7-ClK may provide sufficient neuroprotection to improve neurobehavioural outcome after transient forebrain ischemia in rats.
The experiments in this thesis were undertaken to accomplish two objectives. The primary objective was to develop a rat model of ischemia-induced amnesia that would allow direct comparisons between rats, monkeys and humans, both in terms of ischemia-induced memory deficits, and in terms of their neuropathological basis. This objective was achieved in the Experiments described in Chapters 2 and 3, which provide descriptions of neuropathological and neurobehavioural consequences of transient forebrain ischemia in rats analogous to those that have been reported for monkeys (Bachevalier & Mishkin, 1989; Zola-Morgan et al., 1992) and humans (Squire et al., 1988, Zola-Morgan et al., 1986). The second objective was to use this rat model of ischemia-induced amnesia as a functional assay to assess the therapeutic potential of a drug acting at the glycine binding site associated with the NMDA receptor. This was achieved in the experiments in Chapter 4, which assessed the potential of a glycine site antagonist to mitigate both the ischemia-induced histological changes and the memory deficits in rats characterised in Chapters 2 and 3.

This General Discussion will begin by comparing the rat model of ischemia-induced amnesia developed in this thesis with previous descriptions of ischemia-induced amnesia in rats, monkeys, and humans, in terms of both the neuropathological and the neurobehavioural consequences of ischemia. This will be followed by a discussion of the presumed neuropathological basis of ischemia-induced amnesia. Finally, the potential of the glycine-site on the NMDA receptor complex as a target for pharmacological intervention in transient forebrain ischemia, and the relevance of behavioural testing in ischemia will be discussed.
In order to draw conclusions from animal models concerning the anatomical basis of ischemia-induced amnesia, it is necessary to assess the pattern and extent of the neuropathological changes that are associated with this phenomenon. Therefore, an important component of a rat model of ischemia-induced amnesia is a method for producing transient forebrain ischemia in rats that results in a pattern of brain damage similar to that associated with ischemia-induced amnesia in humans.

The bilateral carotid occlusion method used in our rat model of ischemia-induced amnesia produced extensive loss of CA1 pyramidal neurons in the absence of any significant detectable loss of CA2, CA3 or CA4 pyramidal neurons, or of dentate granule cells. There was some evidence of dentate hilar neuron loss (Experiment 3.2), but this was not consistent. The extent of CA1 cell loss varied along the septotemporal extent of the hippocampus, with the least cell loss occurring at the temporal pole. No cell loss was detected in any of several extrahippocampal structures which are either known to be vulnerable to ischemia (e.g. striatum), or which have been implicated in amnesia (e.g. entorhinal and perirhinal cortices). However, as mentioned above, some cell loss may have occurred in these structures, but not have been detected.

This pattern of cell loss is similar to that seen in the amnesic patient R.B. A prominent feature of the neuropathology of R.B. was a circumscribed bilateral lesion involving CA1, with no other significant damage within the hippocampus. He also suffered minor pathology outside the hippocampus, but this occurred in structures that could not reasonably be associated with his memory impairment (Zola-Morgan et al., 1986). It is also similar to the pattern of cell loss produced in the monkey models of ischemia-induced amnesia. For example, in one study, memory deficits were associated
with significant loss of CA1 and CA2 pyramidal cells, and of somatostatin-containing hilar cells, with no observable extrahippocampal cell loss (Zola-Morgan et al., 1992). Moreover, in this monkey model, the CA1 cell loss was more pronounced in the septal portion of the hippocampus.

From the correspondence between the pattern of cell loss produced by this bilateral carotid occlusion method in rats, and the cell loss observed in R.B., and in monkey models of ischemia-induced amnesia, we inferred that this model of ischemia may be appropriate as a component of a rat model of ischemia-induced amnesia. Moreover, it would appear to be more appropriate than the four vessel occlusion model which has been used in previous studies of ischemia-induced amnesia (e.g. Davis & Volpe, 1990), as it does not produce observable damage within the striatum or other extrahippocampal structures which were not included in R.B.'s lesion. The similarities between the patient R.B., our rat model of ischemia-induced amnesia, and the monkey models of ischemia-induced amnesia at the level of neuropathology enable comparisons between species concerning the anatomical basis of ischemia-induced behavioural changes. The implications of these comparisons will be discussed later.

**ISCHEMIA-INDUCED AMNESIA IN RATS, MONKEYS AND HUMANS**

**Comparison of the recognition memory performance of rats, monkeys and humans following ischemia**

The experiments in Chapter 3 provide a description of the performance of rats on nonspatial object recognition tasks that are thought to be analogous to tasks on which humans (Squire et al., 1988), and monkeys (Bachevalier & Mishkin, 1989;
Squire et al, 1992) show impairments following transient forebrain ischemia. The
demonstration of ischemia-induced deficits on the performance of these tasks by rats
allows direct comparison of the behavioural consequences of ischemia in rats, monkeys
and humans. This, in turn, enables cross-species comparisons of the neuropathological
basis of ischemia-induced memory deficits.

Following ischemia rats were impaired both on a one-trial object recognition
task based on the spontaneous exploration of novel objects (Experiment 3.1) and on a
DNMS task designed to mimic the monkey DNMS task (Experiments 3.2 and 3.3).
The impairments on the one-trial object recognition task are consistent with a
recognition memory deficit following ischemia, as the ability to discriminate between a
novel object and a familiar object on the basis of novelty depends on recognising that
one of the objects is familiar. That is, rats must remember the object to which they
have previously been exposed. However, the performance of ischemic rats on this task
may reflect disruption of cognitive processes other than memory, such as stimulus
preference (which underlies the normal response to novelty), or processes involved in
habituation. These processes are not thought to be disrupted in amnesic patients.
Clearly a recognition memory deficit would be consistent with reports of recognition
memory deficits following ischemia in monkeys and humans. However, as the primary
objective of this thesis is to provide a rat model of ischemia-induced amnesia in which
the behavioural deficits are directly comparable to those seen in humans, the utility of
this task is limited unless further experiments are performed to rule out these alternative
explanations.

The DNMS impairments observed in rats following transient forebrain ischemia
can be compared directly to those that have been reported in nonhuman primates
(Bachevalier & Mishkin 1989; Zola-Morgan et al., 1992), and in humans (Squire, Zola-Morgan & Chen, 1988), as the DNMS tasks used to test rats, monkeys, and humans are essentially equivalent. In each case, correct performance requires that the subjects remember an object that they have encountered previously. Experiments with intact rats suggest that accurate performance on DNMS involves explicit memory for the sample object, as distraction has similar effects on DNMS performance by rats, and on the performance of explicit memory tasks (but not implicit memory tasks) by human subjects.

Ischemic rats were significantly impaired on DNMS compared to controls, both in learning the task and in their subsequent test performance across all retention delays that were studied (Experiment 3.2). Furthermore, extensive presurgery training on DNMS did not prevent or reduce the impairment (Experiment 3.3). The results of Experiment 3.2 complement the results of Zola-Morgan et al. (1992) in which ischemic monkeys that had received no training prior to surgery were tested on DNMS. Ischemic monkeys were impaired at delays of 15 s, 60 s and 10 min, but not at the the shortest delay of 8 s. This is similar to the profile of ischemic rats in Experiment 3.2, who were impaired at all delays tested. However, unlike ischemic rats, ischemic monkeys showed no acquisition impairments on DNMS. In fact, they required fewer trials than controls to reach criterion at the shortest delay, whereas in the present study ischemic rats took significantly more trials to reach criterion than controls. However, when ischemic monkeys were retested on DNMS 18 months after surgery they were impaired in their ability to relearn the basic task. The difference in acquisition of DNMS by ischemic rats and monkeys is not readily attributable to differences in the pattern of damage; selective loss of CA1 and dentate hilar neurons appears to be similar across the two
species. One explanation for the observed difference is that rats and monkeys may use different strategies (which involve different brain structures) to learn the DNMS task, and that the hippocampal damage induced by ischemia consequently disrupts rats more than monkeys. Alternatively, anatomical differences between the limbic structures of rats and monkeys may result in slightly different functional consequences of ischemia. However, there is no direct evidence for either of these possibilities.

The pattern of the behavioural results of Experiment 3.3 is similar to that reported by Bachevalier and Mishkin (1989), who found that ischemic monkeys that had received extensive presurgery training on DNMS took significantly more trials to reattain criterion at a 10-s retention interval and, once having relearned the task, were significantly impaired at each retention interval (10, 30, 60, and 120 s). Furthermore, Bachevalier and Mishkin found that the ischemic monkeys with the most CA1 cell loss displayed the greatest DNMS deficit, a finding that was confirmed by both of the present experiments.

The performance of rats on DNMS following ischemia is also similar to that of humans with ischemia-induced amnesia on the same task (Squire et al., 1988). Human amnesics required more trials to learn the DNMS task, and having achieved criterion at a short retention interval, they were impaired at longer retention intervals. Moreover, the DNMS performance of patients with ischemia-induced amnesia is consistent with their memory deficits on more conventional memory tasks used in neuropsychological assessment, such as word recognition and recall, suggesting that DNMS performance may be a useful indicator of the integrity of the memory system that is disrupted following ischemia. Therefore the similarities, both between the demands of the DNMS tasks used to test humans and rats, and between the ischemia-induced performance
deficits in humans and rats, provide support for the utility of this rat model of ischemia-induced amnesia.

Relation of ischemia-induced nonspatial and spatial memory deficits in rats.

The results of the experiments in Chapter 3 also complement those of several studies that have demonstrated impaired performance on spatial memory tasks following ischemia in rats (Auer et al., 1989; Davis & Volpe, 1990; Hagan & Beaughard, 1990; Jaspers et al., 1990; Kiyota et al., 1991: Ordy et al., 1988), by extending the description of impairments following ischemia to nonspatial recognition memory tasks. The relation between the findings from these studies is not clear, although there are several parallels between the performance of ischemic rats on spatial and nonspatial tasks.

As discussed above, the effects of ischemia on the performance of the one-trial object-recognition task in experiment 3.1 may not reflect a memory impairment. However, a memory deficit on this task would be consistent with the reports of ischemia-induced working memory deficits in rats on spatial tasks (e.g. Volpe et al., 1984), as this task has a working memory requirement, but no obvious reference memory requirement (Ennaceur & Delacour, 1988). Similarly, the DNMS performance of rats following ischemia may be comparable to the spatial memory deficits previously reported. For example, the initial acquisition of trial invariant information required for reference performance on spatial tasks is impaired following ischemia, although rats are still able eventually to acquire this information (e.g. Volpe et al., 1984; Davis et al., 1986). The impaired acquisition of DNMS at a 4-s delay by ischemic rats with no presurgery training may reflect a similar disruption of the ability to acquire trial-
invariant information such as the DNMS rule. However, the impaired reacquisition of DNMS by rats that received extensive presurgery training is not consistent with this explanation, as rats are not impaired on the reference aspect of spatial tasks learned prior to ischemia (Davis, Baranowski et al., 1986). A more plausible explanation for impaired acquisition (and reacquisition) of DNMS following ischemia is that it reflects a working memory impairment: that is, impaired memory for new and variable information. The persistent impairments on DNMS at all of the delays tested may also reflect a working memory deficit, as each trial of DNMS requires the acquisition of new and variable information. Therefore impaired DNMS performance may be analogous to the working memory deficits on spatial tasks seen following ischemia. Another parallel between the performance of rats on spatial tasks and on DNMS is the effect of delays on performance. Ischemic rats are impaired relative to controls at all delays tested although, like control rats, they tend to perform worse at longer delays.

In summary, the results of the present experiments are consistent with those from studies examining spatial-memory performance in rats following transient forebrain ischemia. However, it is not clear that performance deficits on spatial tasks and nonspatial tasks reflect disruption of similar processes.

**Conclusions**

Rats, monkeys and humans are all impaired on recognition memory tasks following ischemic brain damage. Rats are also impaired on spatial memory tasks, although the relation of ischemia-induced spatial and nonspatial memory impairments is not clear. The continuity between rats, monkeys, and humans both in terms of ischemia-induced nonspatial recognition memory deficits, and in terms of ischemia-
induced neuronal loss (discussed above), indicate that the rat model of ischemia-induced amnesia developed in this thesis may be useful, both in the investigation into the neuropathological basis of ischemia-induced memory recognition deficits, and in testing the neuroprotective potential of anti-ischemic drugs. Both of these issues are discussed below.

**WHAT IS THE NEUROPATHOLOGICAL BASIS OF ISCHEMIA-INDUCED AMNESIA?**

Taken together, the histological and behavioural findings of the experiments in Chapters 2 and 3 suggest that ischemia-induced object recognition memory impairments may be attributable to ischemia-induced cell loss within the hippocampal formation. Specifically, extensive loss of CA1 pyramidal neurons, and some loss of dentate hilar neurons appears to be sufficient to produce severe impairments in object recognition memory by rats; ischemia-induced impairments on the object-recognition task and on DNMS were associated with cell loss in these areas, in the absence of any detectable damage in other hippocampal cell fields, or in several other brain areas which either are known to be vulnerable to ischemia (the striatum), or have been implicated in brain-damaged produced amnesia (the mammillary nuclei, and entorhinal and perirhinal cortices). However, it is possible that subtle ischemia-induced neuronal changes such as the loss of a specific subset of neurons, or alterations in the biophysical properties of neurons outside the hippocampus, may have contributed to the memory impairment.

The proposal that partial hippocampal cell loss mediates ischemia-induced memory deficits is consistent with the findings from experiments with monkeys and humans. Both Bachevalier and Mishkin (1989) and Squire et al. (1992) found that the
ischemia-induced memory impairments in monkeys were associated with the loss of CA1 pyramidal neurons and dentate hilar neurons. Monkeys in each of these studies also suffered some extrahippocampal damage, but this was thought to be unlikely to have contributed to the memory deficits, as its location and extent varied between monkeys, and it occurred in brain areas that are not thought to be involved in normal object recognition memory. Similarly, it has been suggested that the only damage suffered by the patient R.B. that could reasonably be associated with his memory deficit was the bilateral loss of CA1 pyramidal neurons (Zola-Morgan, Squire & Amaral, 1986).

Ischemia-induced spatial-working memory deficits in rats are also associated with the loss of CA1 pyramidal neurons and dentate hilar neurons (e.g. Davis & Volpe, 1990, Auer et al., 1989). However, as discussed above, the relation of spatial working memory deficits to nonspatial object recognition memory deficits is not well understood. In addition, the damage induced by the 4VO model of transient forebrain ischemia used in many of the behavioural studies is not restricted to the hippocampus; the dorsolateral striatum consistently suffers cell loss. This additional damage may contribute to the memory deficits found in rats following 4VO, as it has been demonstrated that lesions of the striatum alone can produce deficits on some memory related tasks (Packard, Hirsh & White, 1989). Indeed, it has been suggested that the striatum may be an integral component of a memory system that is anatomically distinct from that which is typically damaged in amnesia (Packard et al., 1989). That is, the striatum may be involved in certain processes that underly nondeclarative memory. Consequently, both the choice of tasks used to assess memory, and the pattern of
damage produced by 4VO in rats, make the results from these experiments difficult to compare with those from human and monkey studies.

In summary, the results of the experiments in this thesis are consistent with the proposal, based on evidence from humans and monkeys, that ischemia-induced recognition memory deficits are mediated by damage within the hippocampus (Zola-Morgan et al., 1986). This proposal is, in turn, consistent with the findings of several experiments showing that large hippocampal lesions produce effects on the performance of memory tasks by rats and monkeys that are similar to those produced by ischemia. For example, both electrolytic lesions (Thomas & Gash, 1988) and neurotoxin lesions (Davis & Volpe, 1990) of the hippocampus result in spatial working memory deficits in rats similar to those seen following ischemia. In addition, complete fimbria-fornix lesions have been shown to impair the performance of rats both on a spatial task (Olton & Papas, 1979), and on a nonspatial delayed matching-to-sample task (Raffaele & Olton, 1988). Similarly, large lesions of the hippocampus have been shown in several studies to result in severe DNMS deficits by monkeys (Mahut, Zola-Morgan, & Moss, 1982; Zola-Morgan & Squire, 1986). Based on the similarities between ischemia-induced memory deficits and the memory deficits that result from large lesions of the hippocampus, it has been proposed that partial hippocampal cell loss induced by ischemia may be functionally equivalent to lesions of the entire hippocampal formation (Zola-Morgan et al., 1992; Davis & Volpe, 1990). This suggestion is supported by analysis of the intrinsic connections of the hippocampus. A useful heuristic in considering hippocampal function has been to consider that the fields of the hippocampal formation are linked by largely unidirectional connections: the perforant path projects from entorhinal cortex to the dentate granule cells; the dentate granule
cells project via the mossy fibres to the CA3 pyramidal cells; the CA3 pyramidal cells give rise to Schaffer collaterals that project to CA1 pyramidal cells. The CA1 pyramidal cells in turn project via the alveus both medially, to the subiculum, and laterally, to the fimbria. Therefore, ischemia-induced loss of CA1 pyramidal cells would effectively prevent transmission through the hippocampus.

However, more recent behavioural and anatomical evidence is not consistent with the proposal that ischemia-induced hippocampal cell loss is functionally equivalent to complete hippocampal ablation. First, large aspiration lesions of the hippocampal formation and overlying posterior cortex do not produce significant DNMS deficits in preoperatively-trained rats except at long (i.e. 10-min) delays (Mumby, Wood & Pinel, 1992), whereas in Experiments 3.2 and 3.3, ischemia-induced CA1 cell loss resulted in severe impairments on the same task. Negative effects of large neurotoxic lesions (Sutherland & McDonald, 1990) and aspiration lesions (Aggleton, Hunt, & Rawlins, 1986) of the hippocampus on the performance of a different nonspatial DNMS task in rats have also been reported. Similarly, surgical removal of the monkey hippocampal formation, parahippocampal gyrus, and posterior entorhinal cortex results in less severe impairments on DNMS in preoperatively trained monkeys than does ischemia-induced damage to CA1 and CA2 (Mishkin, 1978; Bachevalier & Mishkin, 1989). These findings are somewhat paradoxical; they suggest that partial lesions of the rat hippocampus produced by ischemia cause greater functional deficits than complete lesions of the rat hippocampus.

Bachevalier and Mishkin (1989) suggested several possible explanations for the unexpected results in monkeys, two of which can also be applied to the present findings with rats. First, transient forebrain ischemia may induce neuronal loss or functional
changes outside the hippocampus that contribute to the ischemia-induced impairment on DNMS, but remain undetected. As discussed earlier, this possibility cannot be excluded, as the histological techniques used to quantify damage would only have detected substantial cell loss, and not the loss of a small subset of neurons in a given area, or other ischemia-induced functional changes. In order for such extrahippocampal changes to affect performance on DNMS, they would most likely have to occur in circuits which are involved in normal performance on DNMS. Possible loci include the parahippocampal and perirhinal cortices, as substantial evidence has accumulated showing that, at least in monkeys, lesions of the these areas produce severe impairments on DNMS (Murray, Bachevalier & Mishkin, 1989; Meunier, Murray, Bachevalier & Mishkin, 1990; Zola-Morgan, Squire, Amaral, & Suzuki, 1989). An alternative explanation for the paradoxical results is that partial damage to the hippocampus may have resulted in disorganization of the functional activity in the spared cells of the hippocampus and this may, in turn, have disrupted the activity of neurons outside the hippocampus that are involved in some aspect of DNMS. The entorhinal cortex, which is a major constituent of the parahippocampal region, is the major source of cortical input to the hippocampus, but it also receives projections from the hippocampus. Similarly, the perirhinal cortex has reciprocal connections with the hippocampus. In general, the afferent fibres to the hippocampus from these cortical areas project primarily to the dentate gyrus, whereas the hippocampal efferents projecting to the parahippocampal region originate primarily from the subiculum and CA1 (Witter, Groenewegen, Lopes da Silva & Lohman, 1989). It is therefore quite likely that ischemia would result in disorganised activity within the parahippocampal region as a consequence of CA1 pyramidal cell loss. This
may be as, or more debilitating than total hippocampal ablation, as the parahippocampal region projects not only to the hippocampus, but to many telencephalic cortical and subcortical structures (Swanson & Kohler, 1986; Witter et al, 1989). For example, the perirhinal cortex is reciprocally connected to temporal association cortices and the association nuclei of the thalamus.

This explanation would appear to be inconsistent with the view that information flow through the hippocampus is unidirectional, as CA1 cell loss should interrupt all efferent activity. However, the CA1 pyramidal cells are not the only projection neurons of the hippocampus; the CA3 pyramidal cells are thought to project to several areas including the subiculum, the entorhinal cortex and the lateral septum (Swanson, Wyss, & Cowan, 1978; Swanson, Sawchenko & Cowan, 1980), although this claim is challenged by recent anatomical evidence (Ishizuka, Weber & Amaral, 1990). In addition, complete CA1 cell loss is rarely seen following ischemia. Therefore, it is possible that ischemia-induced CA1 cell loss results in the disorganisation of hippocampal activity, and that the consequences of this are functionally dissimilar to those of large aspiration lesions of the hippocampus. The loss of dentate hilar neurons may also contribute to the alterations in hippocampal function following ischemia. The somatostatin immunoreactive hilar neurons, which are the most susceptible in ischemia, project primarily along the septotemporal axis of the hippocampus to terminate on dentate granule cells, suggesting that they may therefore be involved in the integration of dentate granule cell activity along the length of the hippocampus. Specific loss of these cells could therefore contribute to the disorganisation of hippocampal activity that may occur following ischemia. Alterations in hippocampal function following ischemia-induced lesions are consistent with histopathological, histochemical and
autoradiographical demonstrations of neuronal activity modulation and synaptic rearrangement 100 days after ischemia in rats (Onodera, Aoki, Yae, & Kogure, 1990).

The data presented in this thesis do not directly address these issues, and cannot distinguish between the two explanations proposed above for the paradoxical finding of greater deficits after ischemia-induced damage than after large aspiration lesions of the hippocampus. However, recent behavioural evidence from monkeys and rats may bear on this issue. First, Zola-Morgan et al. (1992) found that monkeys with ischemia-induced lesions of CA1 and dentate hilar neurons were as impaired as, but no more impaired than monkeys with circumscribed hippocampal lesions on a number of tasks including DNMS. Monkeys with larger lesions that included the hippocampus, posterior entorhinal cortex and parahippocampal gyrus (H+ lesion) showed similar performance on DNMS but were more impaired on two other memory tasks. The authors argue that if the ischemic monkeys had suffered significant damage in extrahippocampal areas that are important for memory function, they would be expected to have been as impaired as monkeys with the H+ lesion. Therefore it is unlikely that the ischemic monkeys in this study suffered significant extrahippocampal cell loss that affected memory, and it also appears that ischemia-induced damage within the hippocampus is sufficient to produce memory deficits in monkeys. The second set of behavioural data that bear on this issue are those of Volpe et al. (1992) who compared the performance of rats with either ischemia-induced damage, high dose ibotenic acid lesions of the dorsal hippocampus, or low dose lesions of the dorsal hippocampus, on a delayed spatial discrimination task. Rats exposed to ischemia or high dose ibotenic acid lesions were similarly impaired, whereas those exposed to low dose ibotenic acid showed no impairment relative to controls. The ischemic and high
dose ibotenic acid rats suffered comparable CA1 cell loss (75 - 80%), whereas the low dose ibotenic acid rats suffered less (45%) CA1 cell loss. However, the high dose ibotenic acid rats had extensive cell loss in CA2, CA3 and dentate gyrus, whereas there was much less damage to these areas in ischemic and low dose ibotenic acid rats, and the overall extent of damage to the hippocampus in these two groups was comparable. The comparable performance of ischemic and high dose ibotenic acid rats indicates that the extent of CA1 cell loss is a good indicator of functional impairment, and the authors suggest that the dorsal CA1 cell loss seen in these animals is sufficient to cause a memory deficit. However, the possibility that extrahippocampal damage contributes to the memory deficits of these animals cannot be eliminated. The finding that low dose ibotenic rats were less impaired than high dose ibotenic acid rats demonstrates that partial damage to the hippocampus does not disrupt behaviour more than larger lesions, suggesting that disorganised hippocampal activity does not play a role in impaired spatial memory performance in rats.

In summary, although the precise anatomical basis of ischemia-induced object recognition memory deficits in rats cannot be identified on the basis of the available data, the results of the experiments in this thesis are consistent with either of the two explanations discussed. Furthermore, although it is not clear whether the CA1 pyramidal cell loss induced by ischemia can account for the ischemia-induced memory deficits, the significant correlation between CA1 cells and the performance of object recognition tasks by rats indicates that CA1 cell integrity may be a useful predictor of behavioural integrity following ischemia.
IS THE GLYCINE SITE ASSOCIATED WITH THE NMDA RECEPTOR AN APPROPRIATE TARGET FOR PHARMACOLOGICAL INTERVENTION IN ISCHEMIA?

The results of Experiments 4.1 and 4.2 demonstrate that intraventricular administration of 7-Chlorokynurenic acid—a potent antagonist at the glycine site associated with the NMDA receptor—immediately prior to the onset of ischemia significantly reduces ischemia-induced CA1 pyramidal cell loss. The preliminary behavioural findings in Experiment 4.2 also suggest that the neuroprotection provided by 7-ClK may be sufficient to prevent the ischemia-induced memory impairments in rats. These results are consistent with the excitotoxic hypothesis of ischemic brain damage, as antagonism of the glycine binding site on the NMDA receptor would be expected to decrease NMDA channel opening (Dingledine et al., 1990), thus attenuating glutamate-stimulated calcium entry via the NMDA receptor during ischemia. They also provide evidence that the glycine binding site on the NMDA receptor-channel complex may be a relevant target for potential anti-ischemic drugs.

Several lines of reasoning are consistent with the glycine binding site on the NMDA receptor being a more appropriate target for drug intervention in ischemia than the glutamate binding site, or the ion channel itself. First, the degree of protection provided by 7-ClK is similar to that reported for the competitive NMDA antagonist AP7 against CA1 cell loss induced by 10 min bilateral carotid occlusion combined with hypotension (Swan, Evan & Meldrum, 1988), and for the non-competitive NMDA antagonist MK801 against CA1 cell loss induced by 5 min bilateral carotid occlusion and hypotension (Gill, Foster, & Woodruff, 1987; Gill & Woodruff, 1990). Therefore, it appears that 7-Chlorokynurenic acid is at least as effective as these drugs. Second,
noncompetitive NMDA antagonists such as MK801 and ketamine are effective only at
doses that produce profound behavioural disturbances, whereas there is no evidence
that 7-ClK produces detrimental side effects of this nature\(^3\). The behavioural changes
that have been associated with this drug, such as deficits on a spatial memory task by
rats (Bannerman, Butcher, & Morris, 1991), reduced spinal nociception in rats
(Dickenson & Aydar, 1991) and a decrease in seizure sensitivity in amygdala kindled
rats (Croucher & Bradford, 1991) are thought to be a direct result of attenuating
NMDA channel activity, as opposed to non-NMDA mediated side effects.
Furthermore, both 7ClK and HA-966 (another glycine site antagonist) blocked NMDA-
induced convulsions in mice without producing PCP-like behavioural effects
(locomotion and falling), whereas the ability of noncompetitive NMDA antagonists
and, to a lesser extent, competitive NMDA antagonists, to block NMDA-induced
convulsions was significantly correlated with the production of PCP-like behaviours
(Koek & Colpaert, 1989). Third, there is no evidence that 7-ClK binding is sensitive to
membrane voltage, whereas the ability of the noncompetitive NMDA antagonists to
block the channel is compromised when the cell membrane markedly depolarised
(Miller, 1987). Fourth, elevated levels of glutamate do not affect the ability of 7-CLK
to prevent NMDA channel opening, whereas the inhibitory effects of competitive
NMDA antagonists can be overcome by high levels of glutamate (Kloog, Lamdani-Itkin
& Sokolovsky, 1990). Finally, as discussed earlier, elevated extracellular levels of
glycine during ischemia may potentiate the NMDA-channel opening during ischemia
increasing glutamate binding (Fadda, Danysz, Wroblewski & Costa, 1988) perhaps by

\(^3\)This claim may be premature, as few studies have examined the effects of 7ClK in vivo.
accelerating the recovery of the NMDA receptor from a desensitised state (Mayer et al., 1989).

In conclusion, the available data support the proposal that the glycine binding site associated with the NMDA receptor may be an appropriate target for pharmacological intervention in ischemia. The behavioural data in Experiment 4.2 provide additional support for this claim, as they indicate that the neuroprotection provided by 7-ClK may be sufficient to improve behavioural outcome following ischemia. However, there are many issues that were not addressed in the present experiments that may have important implications for the use of glycine site antagonists in vivo. For example, we did not quantify cells in retrosplenial or posterior cingulate cortex, leaving open the possibility that 7-ClK, like the noncompetitive and competitive NMDA antagonists, causes neurotoxic side effects in these areas. In addition, we did not investigate the time window or the range of doses at which 7-ClK is efficacious. However, previous findings with NMDA antagonists indicate that 7CIK and other glycine site antagonists may reduce neuronal death only if administered in the early stages of ischemia. Another limiting factor may be the ability of 7-ClK to permeate the brain following systemic administration. Finally, the findings with 7CLK may not extend to HA-966 which, although preventing the potentiation of NMDA responses by glycine, has partial agonist activity at the glycine site (Kemp & Priestley, 1991), and is also affected by NMDA agonist binding (Kloog, Lamdani-Itkin & Sokolovsky, 1990).

In an earlier section it was suggested that, as brain function is the main concern of patients surviving transient ischemic episodes, it is essential to assess the neurobehavioural efficacy, as well as the neuropathological efficacy, of potential anti-ischemic drugs. Experiment 4.2 provides preliminary evidence that, in the case of 7-
CIK, neuroprotection is accompanied by an improvement in behavioural outcome. Moreover, a consistent result in each of the behavioural studies in this thesis was that the magnitude of the behavioural impairment was related to the degree of CA1 pyramidal cell loss. Rats with the fewest CA1 pyramidal cells were the most impaired on object recognition tasks in each of the experiments. Taken together, these findings suggest that CA1 cell number is a useful predictor of performance on memory tasks following ischemia. However, as discussed earlier, CA1 cell loss may not mediate the memory impairments. Rather, extrahippocampal cell loss, or functional changes that cannot be observed histologically, may be responsible for the observed behavioural impairments. It is also possible that the improvement in neurobehavioural outcome provided by 7-CIK may be due to neuroprotection to cells other than CA1 pyramidal neurons. In this context, a significant finding is that 24 min bilateral carotid occlusion with no hypotension in rats results in impaired water maze performance, but no detectable neuronal necrosis (Jaspers et al, 1990). Therefore, until the precise anatomical basis of ischemia-induced memory impairments has been identified, it is essential to use behavioural measures to assess neuroprotection in ischemia.

CONCLUSIONS

The main theme of this thesis has been to develop a rat model of ischemia-induced amnesia that incorporates both neuropathological and behavioural features of ischemia-induced amnesia in the clinical population. The interdisciplinary approach used in the characterisation of ischemia-induced amnesia in this rat model has allowed investigation into the neuropathological basis of ischemia-induced memory impairments, and has enabled preliminary assessment of the therapeutic potential of a
glycine receptor antagonist in ischemia, both at an anatomical level and at a behavioural level.
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