Electrophysiological Actions of Hemoglobin on CA1 Hippocampal Neurons

by

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Date 11th July 94

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Hemoglobin, the oxygen-carrying component of red blood cells, is known as a nitric oxide (NO) chelating agent. For this reason, hemoglobin has been used widely in studying the role of nitric oxide in long-term potentiation (LTP) and excitotoxicity. However, the direct electrophysiological actions of hemoglobin has not been examined. In this investigation, the actions of hemoglobin on rat hippocampal CA1 neurons were studied since hemoglobin may be present in hemorrhagic stroke and other head injuries. Superfusion of rat hippocampal slices with 0.1 mM of bovine hemoglobin for 15 minutes was induced a significant depolarization associated with an increase in the input resistance. In addition, hemoglobin suppressed the evoked synaptic responses and increased the depolarization-induced discharge of action potentials, of rat hippocampal CA1 neurons. These hemoglobin-mediated changes usually recovered partially 30 minutes after the removal of hemoglobin.

While the depolarizing action of hemoglobin was enhanced in a calcium-free medium, it was not significantly changed by 2-amino-5-phosphonovalerate (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). These observations suggest that the depolarizing action of hemoglobin is independent of the presence of extracellular calcium and activations of the excitatory amino acid receptors. Because hemoglobin has been observed to suppress the depolarizing action of glutamate, it is possible that hemoglobin suppresses the EPSP by interfering with the actions of glutamate. Although hemoglobin has been suggested to suppress LTP and excitability by scavenging nitric oxide (Garthwaite et al., 1988; Haley et al., 1992; O’ Dell et al., 1991; Schuman and Madison, 1991), the reported actions of hemoglobin were not removed by pre-treatment with 100 μM or 500 μM of Nω-nitro-L-arginine, a nitric oxide synthase inhibitor. Similar to the
scavenging property of hemoglobin, the iron content of hemoglobin probably did not contribute to the actions of hemoglobin since 0.4 mM or 2.0 mM of ferric chloride did not simulate the effects of hemoglobin.

Because neurons can be exposed to hemoglobin in hemorrhagic stroke and head injuries, the electrophysiological actions of hemoglobin on rat hippocampal CA1 neurons may be relevant to the neurological complications associated with intracranial hemorrhage and head injuries. Further studies on mechanisms of the electrophysiological actions of hemoglobin are necessary for understanding the role of hemoglobin in neuronal damages associated with hemorrhagic stroke and other head injuries.

Bhagavatula R. Sastry
(Supervisor)
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<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>AHP</td>
<td>Afterhyperpolarization</td>
</tr>
<tr>
<td>AP3</td>
<td>2-amino-phosphonopropionic acid</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-phosphonovalerate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu ammonis</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3':5'-cyclic phosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3':5'-cyclic phosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
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<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
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<tr>
<td>CO</td>
<td>Carbonmonoxide</td>
</tr>
<tr>
<td>DNQX</td>
<td>6,7-dinitroquinoxaline-2,3-dione</td>
</tr>
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<td>DTX</td>
<td>Dendrotoxin</td>
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<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
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<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>GABA-T</td>
<td>GABA transaminase</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>L-AP4</td>
<td>L-2-amino-4-phosphonobutanoic acid</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>LEA</td>
<td>Lateral entorhinal area</td>
</tr>
<tr>
<td>MEA</td>
<td>Medial entorhinal area</td>
</tr>
<tr>
<td>MK801</td>
<td>Dizolcipine</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(F)quinoxaline</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
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<td>RMP</td>
<td>Resting membrane potential</td>
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<td>TEA</td>
<td>Tetraethylammonium</td>
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<tr>
<td>trans-ACPD</td>
<td>trans-1-amino-1,3-cyclopentanedicarboxylic acid</td>
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<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
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DEDICATION

Dedicated to my parents
1) INTRODUCTION:

Cerebrovascular diseases and head injuries are often associated with neurological complications. Dementia associated with loss of learning and memory is sometimes observed as a consequence of cerebrovascular diseases and head injuries. In hemorrhagic stroke and cerebrovascular injuries, extravascular pooling of blood in the surrounding brain tissue occurs. Since the intracranially pooled blood takes hours to days to disappear (Sornás et al., 1972), the brain tissue is exposed to an abnormal environment for prolonged periods of time. As a consequence, hemoglobin may leak out of erythrocytes and neurons may be exposed to this substance. The mechanisms involved in the above mentioned neurological complications are unclear. If, in fact, neurons are exposed to hemoglobin for prolonged periods of time, the possibility that this agent is a contributing factor to neuronal damages associated with stroke and other cerebrovascular diseases, should be examined.

NO has been suggested as a diffusible messenger which links activation of postsynaptic NMDA receptors to functional modifications in neighboring presynaptic terminals and glial cells (Garthwaite et al, 1988). Since hemoglobin is known as a NO scavenger which is not membrane-permeant (Gibson and Roughton, 1957), this agent has been used in studying the role of NO in excitotoxicity and synaptic plasticity. Hemoglobin has been found to suppress LTP (Haley, 1992; Musleh, 1993; O'Dell, 1991; Schuman and Madison, 1991) as well as the population EPSP (Garthwaite et al., 1988). However, the role of NO in LTP and in excitotoxicity is controversial (Izumi et al., 1992b; Pauwels and Leysen, 1992). Even though hemoglobin has been used widely as a NO chelator in studying the role of NO, the direct electrophysiological actions of hemoglobin on neurons have not been examined. It is possible that mechanisms besides chelation of
NO may be involved in suppression of the population EPSP, LTP, and excitotoxicity. Moreover, these unknown actions of hemoglobin may contribute to neuronal deficits induced by hemorrhagic stroke and cerebrovascular injuries. In this study the electrophysiological effects of hemoglobin on rat hippocampal CA1 neurons were examined. It is hoped that information from these studies will form a basis to determine if the agent is related to neurological complications associated with hemorrhagic stroke and other cerebrovascular injuries.
2) THE HIPPOCAMPAL FORMATION:

2.1) Development of the Hippocampal Formation:

During ontogenic development, the cortical mantle is subdivided into the allocortex and the isocortex. While the isocortex separates completely from the cortical mantle to form the neocortex, the allocortex which contains the archicortex and the paleocortex remains attached to the cortical mantle (Filimonoff, 1947). The archicortex or hippocampus consists of the subicular complex, the hippocampus proper (Ammon's horn), the dentate gyrus (fascia dentata), and both precommissural and supracommissural hippocampi (Chronister and White, 1975; Schwerdtfeger, 1984; Teyler and DiSenna, 1984). The paleocortex contains the olfactory bulb, the accessory olfactory bulb, the anterior olfactory nucleus, the olfactory tubercle, the periamygdaloid region, the septum, the diagonal region, and the periform region (Schwerdtfeger, 1984).

Besides the archicortex and the paleocortex, an additional structure denoted as the periallocortex exists between the allocortex and the isocortex. The periallocortex comprises of the entorhinal, the peripaleocortical, the claustral, the presubicircular, the retrosplenial, and the periarchicortical cingulate cortices (Blackstad, 1956; Brodmann, 1909; Chronister and White, 1975; Lorente De Nó, 1934; Sandies, 1972; Vaz Ferreira, 1951, White, 1959).

2.2) Nomenclature and Architectonics of the Rat Hippocampus:

The rat hippocampus, a bilaterally symmetrical structure which resembles a cashew nut in shape, extends from the septal nuclei rostro-dorsally to the temporal lobe caudo-ventrally (Green, 1964; Teyler and DiScenna, 1984). The hippocampus is situated below the neocortex and along the floor of the descending horn of the lateral
ventricle. The longitudinal or septo-temporal axis of the hippocampus runs from the septal pole to the temporal pole while the transverse axis is perpendicular to the longitudinal axis (Fig. 1).

The hippocampus is composed of the Ammon's horn, the dentate gyrus, and the subiculum. The ventricular surface of the hippocampus is covered by a white fiber layer, the alveus. These fibers, which are mainly constituted by axons from cells of the Ammon's horn, converge to form the fimbria on the medial surface of the hippocampus (Teyler and DiScenna, 1984). The granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon's horn are the two principal layers in the rat hippocampus.

Based on Golgi preparations, Ramón y Cajal (1911) subdivided Ammon's horn into an area of large pyramidal cells (regio inferior) and a region of more densely packed smaller pyramidal cells (regio superior). Unlike Ramón y Cajal, Lorente de Nó (1934) divided Ammon's horn or hippocampus proper into four subfields, CA1 to CA4. Although CA4 in Lorente de Nó's scheme corresponds to the polymorphic zone of the dentate gyrus described by Ramón y Cajal, it is unclear as to whether the CA4 region belongs to the dentate gyrus or the hippocampus proper. More recent studies tend to support that the polymorphic zone belongs to the dentate gyrus rather than to the Ammon's horn (Blackstad, 1956; Amaral, 1978; Gaaraskjaer, 1981). While areas CA2 and CA3 together correspond to the regio inferior, area CA1 resembles the regio superior.
Figure 1. Orientation of the rat hippocampus. While the septotemporal axis runs from the septal pole to the temporal pole, the proximodistal axis runs from the hilus of the dentate gyrus toward the subiculum. Adapted from Witter (1989).
2.2.1) The Dentate Gyrus:

In the dentate gyrus, granule cells, which are ovoid in shape and 15 to 25 μm in diameter, are the major cell type in the cell layer, stratum granulosum (Golgi, 1886). The granule cell layer can be categorized, in relation to its location to the pyramidal cells of CA3, into a suprapyramidal (upper) and an infrapyramidal (lower) blade which merge at the crest of the dentate gyrus. While the blade which is adjacent to the hippocampal fissure is called the suprapyramidal blade, the opposite blade is the infrapyramidal blade (Chronister and White, 1975; Swanson et al., 1978). As a result of the sharp curvature of the dentate gyrus, the dentate gyrus contains the hilar area which is composed of several layers of polymorphic cells (Amaral, 1978; Lorente de Nó, 1934).

There are three strata, namely the stratum granulosum, the stratum moleculare, and the stratum polymorphe, in the dentate gyrus. While the stratum granulosum is populated by granule cells, the stratum moleculare is where the perforant path projections terminate. The stratum polymorphe contains various cell types such as basket and modified pyramidal cells which give rise to at least two systems of associational connections that end within the dentate gyrus. The axons of granule cells, mossy fibers, collateralize in the polymorphic cell layer before entering the CA3 field where they form en passant synapses on the proximal dendrites of the pyramidal cells (Blackstad et al., 1970; Clai borne et al., 1986; Gaarskjaer, 1978; Gaarskjaer, 1986), whereas the dendrites project into the stratum moleculare layer.
2.2.2 The Hippocampal Proper:

Following the pyramidal cell layer of Ammon's horn along its transverse axis from the dentate gyrus (proximal) to the subiculum (distal), the CA3 field merges distally with the CA2 field; the proximal part of CA1 joins CA2; and the distal part of CA1 connects the subiculum. The CA2 subfield which is not innervated by mossy fibers is a transition zone between the CA1 and CA3 subfields (Chronister and White, 1975; Lorente de Nó, 1934). The CA1 and CA3 subfields are further categorized into CA1a, CA1b, CA1c, CA3a, CA3b, and CA3c (Lorente de Nó, 1934). The principle cell type in Ammon's horn is the pyramidal cell which is pear in shape and 25 to 40 μm in diameter (Golgi, 1886).

In addition to the pyramidal cell layer or stratum pyramidale, the alveus which lies next to the epithelium of the lateral ventricle, the stratum oriens which is situated between the alveus and the stratum pyramidale, the stratum lucidum which is next to the stratum pyramidale, the stratum radiatum, the stratum lacunosum, and stratum moleculare, can also be found in the Ammon's horn (Kolliker, 1896; Cajal, 1911; Lorente de Nó, 1934). While the stratum alveus contains primary efferent axons of pyramidal cells, the stratum oriens comprises of the basal dendrites of pyramidal cells, axons originating from the alveus, and non-pyramidal neurons. The stratum lucidum contains mossy fibers from the dentate gyrus. The stratum pyramidale is occupied by the soma of pyramidal cells. The apical dendrites of pyramidal cells lie in the stratum radiatum. In the stratum lacunosum-moleculare, both interneurons and distal dendrites of the pyramidal cells can be found.

In rat, the separation between the stratum radiatum and the stratum lacunosum is not well defined. Therefore, these two layers are collectively described as the
stratum radiatum (Lorente de Nó, 1934). For a similar reason, the stratum lucidum and the stratum pyramidale are considered as one layer (Lorente de Nó, 1934). The boundaries of the stratum radiatum and the stratum oriens mark the end of the hippocampus proper at the hilus. The abrupt termination of the stratum pyramidale in the CA1 field defines the boundary at the subicular end (Angevine, 1975; Blackstad, 1956; Ramón y Cajal, 1893; Lorente de Nó, 1934).

2.2.3) The Subicular Complex:

The subiculum, which lies between the CA1 subfield of the hippocampus proper and the presubicular region of the perialloccortex, is replaced by the pre- and parasubiculum distally, the retrosplenial cortex dorsally, as well as the entorhinal cortex ventrally. The rat entorhinal cortex, which can be subdivided into LEA and MEA (Blackstad, 1956; Steward, 1976; Wyss, 1981; Krettek and Price, 1977), are constituted by six cortical layers, namely the superficial (layers I-III) and the deep (layers IV-VI) layers.

2.2.4) The Hilus:

Not only do the dentate granular cells send mossy fibers to CA3, but also distribute many axon collaterals within a relatively narrow lamella of the hilus of the dentate gyrus (Claiborne et al., 1986; Fricke and Cowan, 1978; Swanson et al., 1978). Most of the associational and commissural inputs to the dentate gyrus, which terminate in the inner one-third of the molecular layer of the dentate gyrus, arise from hilar cells. The hilar region receives extrinsic afferents predominantly from the brain stem and the septal area. These afferents include a prominent noradrenergic input from the locus coeruleus, a serotonergic input from the median and dorsal raphe, a cholinergic input from the medial septal nucleus, and a histaminergic input from the
supramammillary region. Even though these afferents are most dense in the hilus of the dentate gyrus, innervations of the other subfields of the hippocampus have also been reported (Swanson et al., 1987). Because connections between the dendritic trees of hilar cells and the molecular layer of the dentate gyrus have been suggested (Amaral, 1978), these dendrites may be influenced directly by the major cortical inputs to the dentate gyrus. Therefore, the hilus is probably a functionally different unit of the hippocampus which may play a regulatory role in the dentate gyrus. It may either enhance or inhibit information flow in the dentate gyrus.
3) LAMELLAR ORGANIZATION OF THE HIPPOCAMPUS:

Electrophysiological data have suggested that the hippocampus is organized in a lamellar fashion (Andersen et al., 1971b). When the entorhinal area is stimulated, four pathways, namely the perforant pathway, the mossy fibers, the Schaffer collaterals, and the alvear fibers of CA1 become activated successively (Andersen et al., 1971b). Each lamella is located perpendicularly to the longitudinal axis of the hippocampus. Connections from the dentate gyrus to the subiculum can be found in each lamella. While the dentate gyrus, whose major cortical afferents originated from the entorhinal cortex, is the major input structure of the hippocampus, the subiculum is the main source of the hippocampal efferents to subcortical and cortical areas, including the entorhinal cortex (Witter, 1986; Swanson et al., 1987).

On the contrary, studies conducted by Hjorth-Simersen (1973), Laurberg (1979), and Swanson et al. (1978) suggest that the intrinsic circuitry of the hippocampus extends along the septo-temporal axis as much as in the transverse direction. By using an extended hippocampal preparation (a procedure in which the septal to temporal curvature of the hippocampus is corrected by flattening the hippocampus so that a more accurate representation of fibers being distributed in the septo-temporal plane when transverse slices are cut) and Phaseolus vulgarus leucoagglutinin (PHA-L), the three-dimensional organization of the intrinsic hippocampal circuitry, except mossy fibers, is not restricted to a lamellar array.
4) ORGANIZATION OF THE TRISYNAPTIC CIRCUIT:

Mossy fibers which arise from the dentate granule cells innervate the entire transverse or proximodistal extent of CA3. These highly laminated fibers form synaptic contacts with proximal dendrites of CA3 pyramidal cells. The proximal portions of CA3 preferentially interact with fibers that originate in the infrapyramidal blade, the crest, and the adjacent portion of the suprapyramidal blade (Claiborne et al., 1986). Neurons at the tip of the suprapyramidal blade are linked to more distal portions of the CA3 field. The mossy fibers which terminate on the basal dendrites of more proximally located CA3 pyramidal cells originate principally from the infrapyramidal blade of the dentate gyrus.

Schaffer collaterals, which arise from the pyramidal cells in field CA3, synapse in the stratum radiatum and stratum oriens with the dendrites of CA1 pyramidal cells. The CA1 pyramidal cells in turn give rise to projections to the subiculum which terminate in the deep half of the molecular layer and in the pyramidal cell layer. The connections between CA3 and CA1 and those from CA1 to the subiculum are organized perpendicularly to the cell layer in a columnar manner. Neurons in CA3 and CA1 constitute axonal columns in CA1 and the subiculum, respectively. While fibers from proximal parts of CA3 and CA1 interact with distal parts of CA1 and the subiculum, respectively, more distal parts of CA3 and CA1 are linked to the more proximal parts of CA1 and the subiculum, respectively (Tamamaki et al., 1987; Ishizuka et al., 1988).
5) INTRINSIC CIRCUITRY OF HIPPOCAMPUS:

5.1) Entorhinal cortex and the Perforant Path:

The topographical organization of the entorhinal-dentate projections and that of the afferents of the entorhinal cortex indicate that the lateral and caudal parts of the entorhinal cortex which mediate sensory information project densely to septal and intermediate parts of the dentate gyrus, in particular to the suprapyramidal blade. More medial parts of the entorhinal cortex project preferentially to more temporal levels of the dentate gyrus. These parts of the entorhinal cortex also show a slight preference for the infrapyramidal blade. Because more medial parts of entorhinal cortex differ with respect to their afferents from the lateral part, different subsets of dentate granular cells probably mediate different types of information (Witter et al., 1986, 1989). Projections to Ammon’s horn have also been reported by Steward (1976) and Wyss (1981). While projections to the dentate gyrus and CA3 arise mainly from neurons in layer II, projections to CA1 originate from cells in layer III (Stewart and Scoville, 1976).

5.1.1) The Perforant Pathway:

The entorhinal cortex gives rise to the perforant pathway which projects strongly to the dentate and the hippocampus (Ramón y Cajal, 1893; Lorente de Nó, 1933, 1934; Blackstad, 1956, 1958; Raisman et al., 1965). The fibers of the perforant path travel dorsally and eventually along the transverse plane. They enter the dentate gyrus and the hippocampus after coursing through the pyramidal layer of the subiculum along its long axis. Besides the pyramidal layer of the subiculum, perforant path fibers also distribute within the molecular layer of the dentate gyrus, stratum lacunosum-moleculare of the hippocampus and the molecular layer of the subiculum.
It has been reported that fibers of the perforant path cross the hippocampal fissure at the connection between the suprapyramidal and infrapyramidal blades of the dentate gyrus (i.e. the crest), bifurcate and extend branches that ramify into the suprapyramidal and infrapyramidal blades of the molecular layer (Golgi, 1886).

Although Blackstad (1956) and Hjorth-Dimonsen and Jeune (1972) suggested that the majority of the perforant pathway fibers traverse the molecular layers of the subiculum and CA1, and subsequently cross the hippocampal fissure to reach the molecular layer of the dentate gyrus, Witter et al. (1988) has reported that fibers which follow the above pathway are not significant. Many of the entorhinal fibers travel along the transverse axis of the molecular layer of the Ammon's horn and subsequently enter the suprapyramidal tip of the molecular layer of the dentate gyrus. These observations imply that entorhinal fibers can either reach the dentate gyrus directly or interact with cells in CA3 before reaching the dentate granule cells.

5.1.2) The Lateral and Medial Components of the Perforant Pathway:

The perforant pathway can be divided into two components which arise from different parts of the entorhinal cortex and differ with respect to their distribution in the hippocampus. The lateral perforant pathway is organized in a way which allows a small part of LEA to interact with a large part of the hippocampus along its longitudinal axis and one-third of the apical dendrites of the cells in the dentate gyrus and CA3. While the lateral perforant pathway has been reported to distribute preferentially to the suprapyramidal blade of the dentate gyrus, the medial component either does not show a preference or prefers the infrapyramidal blade of the dentate gyrus (Wyss, 1981). Septal parts of the dentate gyrus are innervated by the lateral perforant
pathway which originates laterally in LEA and the medial perforant pathway which arises from the most caudolateral part of MEA. The more intermediate parts of the dentate gyrus are influenced by the lateral and medial components originating at approximately the same rostrocaudal level in LEA and MEA.

In addition to the projections to the dentate gyrus, the lateral perforant pathway projects to the distal part of CA1, whereas the medial perforant pathway projects to the more proximal portion of CA1. Generally, fibers from LEA distribute more extensively along the longitudinal axis of the hippocampus than do fibers that originate in MEA (Witter et al., 1988).

The difference in organization of the two components of the perforant pathway implies that these two components may exert different actions on the same target neurons in the dentate gyrus and CA3. In the subiculum, they may affect the same cells in the same way even though both components differ with respect to their neuroactive substances and electrophysiological characteristics (McNaughton, 1980; Fredens et al., 1984). In CA1, these two components may influence different cell populations.

5.2) Dentate Gyrus and the Mossy Fibers:

Mossy fibers are principally oriented transverse to the long axis of the hippocampal formation. Mossy fibers arising from the septotemporal level of the dentate gyrus minimally overlap with those originating from other septotemporal levels. After these fibers turn abruptly and caudally at the septal levels of the hippocampal formation as they approach the CA1 field, they travel along the long axis of the hippocampus (Ramón y Cajal, 1893, Swanson et al., 1978). At mid and temporal levels of the hippocampal formation, mossy fibers are not as temporally
directed (i.e. along the long axis) as the septal level. Mossy fibers which originate from the infrapyramidal blade travel in stratum oriens and terminate within subfields CA3b and CA3c, whereas those arising from the suprapyramidal blade travel in the stratum lucidum, course through CA3, and terminate at CA2 (Blackstad et al., 1970; Chronister and White, 1975; Lorente de Nó, 1934). Throughout their course, mossy fibers form excitatory en passant synapses with the dendrites of pyramidal cells in CA3 (Andersen et al., 1966a) In addition to pyramidal cells, mossy fibers also synapse on basket cells and neurons in the hilus of the dentate gyrus. By using PHA-L, the mossy fiber projection to the CA3 field has been reported to demonstrate a somewhat lamellar organization (Amaral and Witter, 1989).

5.3) Dentate Gyrus and the Associational Projection:

Unlike the mossy fiber projection to the CA3 field, the associational projection to the dentate gyrus is not organized in a lamellar fashion. The associational projection to the dentate gyrus arises mainly from neurons in the polymorphic layer of the dentate gyrus, not from the pyramidal cells in CA3 (Hjorth-Simonsen and Laurberg, 1977; Laurberg, 1979; Laurberg and Sorensen, 1981). The inner one-third of the molecular layer of the dentate gyrus is innervated by fibers of ipsilateral origin, namely the cells of the polymorphic region or the CA3 pyramidal cells which are close to the dentate gyrus (Swanson et al., 1978; Zimmer, 1971). This projection appears to be divergent along the long axis of the dentate gyrus (Swanson et al., 1978). The associational projection is not organized to provide feedback to granule cells at the same level in which polymorphic cells are located, but to levels as far as several millimeters away from the cells of origin (Ishizuka et al., 1990).
The polymorphic cells of the dentate gyrus do not project to the Ammon's horn. The associational projections within CA3 and the Schaffer collateral system to CA1 originate exclusively in the CA3 and CA2 fields of the hippocampus (Hjorth-Simonsen and Laurberg, 1977; Laurberg, 1979; Laurberg and Sorensen, 1981).

5.4) Projections Originating from CA3:

The pyramidal cells of CA3 give rise to associational projections that terminate within CA3 and Schaffer collaterals which innervate CA1 (Swanson et al., 1980; Swanson et al., 1981). The associational projections, which travel primarily parallel to the long axis, link different levels of the hippocampus, whereas the Schaffer collaterals only link the CA3 and CA1 fields of the same hippocampal level (Lorente de Nó, 1934). The projections from CA3 to CA3 and from CA3 to CA1 terminate extensively both in the stratum oriens and stratum radiatum, but not in the stratum lacunsum-moleculare (Hjorth-Simonsen, 1973).

Schaffer collaterals course through CA1 and make synaptic contacts with pyramidal cells on their basal dendrites in the stratum oriens and at the proximal three-quarter of the apical dendrites in the stratum radiatum (Gottileb and Cowan, 1973; Lorente de Nó, 1934). Fibers arising from CA3 cells which are close to the dentate gyrus project preferentially to the distal portion of CA1 or where they terminate in the superficial portion of the stratum radiatum. Fibers originating from CA3 cells which are close to the CA1 border distribute predominantly to parts of CA1 that are close to CA3 and to deeper portions of stratum radiatum. In general, CA3 cells located close to the dentate gyrus project preferentially in a septal direction, whereas those which are located near the CA1 border tend to project exclusively in a temporal direction.
5.5) Miscellaneous Intrinsic Connections:

Unlike the CA3 cells, CA1 pyramidal cells do not give rise to projections to other levels of CA1. Fibers from CA1 cells project to the subiculum in a columnar manner (Finch and Babb, 1981; Finch et al., 1983; Tamamaki et al., 1987). Weak projections from CA1 to the deep layers of the entorhinal cortex have also been observed (Swanson et al., 1978).

Other intrinsic connections such as projections from the subiculum to the presubiculum, the parasubiculum, and the entorhinal cortex (Beckstead, 1978; Finch et al.; 1983, Köhler, 1985; Köhler et al., 1978; Shipley, 1975), projections from the presubiculum and parasubiculum to layers III and II of the entorhinal cortex, respectively (Köhler, 1985; Shipley, 1975), and projections in the entorhinal cortex which link various parts of the region and the deep layers with more superficial layers (Köhler 1986; Köhler, 1988; Witter et al., 1986), also exist in the hippocampus.
6) EXTRINSIC CIRCUITRY OF THE HIPPOCAMPUS:

6.1) Extrinsic Hippocampal Afferents:

6.1.1) Septal Projections:

In addition to fibers of the perforant path, fibers which originate in the lateral and medial septal nuclei, as well as the nucleus of the diagonal band project to the dentate gyrus. These septo-hippocampal projections which are primarily cholinergic efferents (Kramis et al., 1975) are responsible for the rhythmic theta activity, which are reflective of a behavioral state corresponding to intentional movement, in the hippocampus (Petsche et al., 1962; Vanderwolf et al., 1975). Because some neurons in the medial septal nucleus and nucleus of the diagonal band are glutamic acid decarboxylase (GAD) positive (Köhler et al., 1984; Panula et al., 1984), it is possible that GABAergic input to the dentate gyrus may exist.

Besides the dentate gyrus, fibers from the medial septal nucleus also project to CA3. These projections which are mainly cholinergic (Frotscher et al., 1986; Houser et al., 1983) concentrate in stratum oriens (Nyakas et al., 1987). Projections from the medial septal nucleus to CA1 are controversial since some investigators (Monmaur and Thompson, 1983; Sakanaka et al., 1980) observed the presence of this pathway whereas others (Powell, 1963; Rose et al., 1976; Swanson and Cowan, 1979) did not.

6.1.2) Isocortical Projections:

Projections from the isocortex to the hippocampus, which arise from the parietal, the temporal, and the perirhinal cortices, terminate exclusively in CA1, occasionally in CA3, and not in the dentate gyrus (Schwertfeger, 1979; Schwertfeger, 1984). Because these projections link the hippocampus with different regions of the cortex, hippocampal function can be influenced by cortical sensory input.
In rats, the entorhinal cortex receives an input from the perirhinal cortex (Köhler, 1986). The perirhinal fibers which receive their input from widespread sensory-related parts of the cortical surface (Deacon et al., 1983) predominantly terminate in lateral portions of LEA and lateral and caudal parts of MEA (Witter et al., 1988). Although sensory convergence has been suggested to take place in the superficial layers of lateral parts of the entorhinal cortex (Vaysettes-Courchay and Sessler, 1983), not much is known with respect to the afferents of more medial parts of the entorhinal cortex. The medial parts of the entorhinal cortex are thought to receive information different from that received by the lateral parts (Witter et al., 1986, 1989).

6.2) Extrinsic Hippocampal Efferents:

The hippocampus projects to various cortical and subcortical structures, including the entorhinal cortex, the septum, the nucleus accumbens, the amygdaloid complex, and the hypothalamus. Although these extrinsic projections originate mostly from the subiculum, Ammon's horn, especially CA1, also contributes to these projections (Swanson et al., 1987). While the projection to the nucleus accumbens originates in the cells that are located at the subicular-CA1 border, the more distally located neurons in the dorsal subiculum projects to the retrosplenial and perirhinal cortices (Groenewegen et al., 1987). Although projections to the nucleus accumbens, and the retrosplenial and perirhinal cortices originated in differently located subicular cell populations, projections to the septum and entorhinal cortex arise from the same neuronal population.
6.2.1) Fornix-Fimbria System:

The fornix-fimbria system is the most well studied extrinsic pathway in the hippocampus (O'Keefe and Nadel, 1978). Efferent fibers from the hippocampus and adjacent allocortical areas converge in the fimbria. These fibers then course through the septo-fimbrial nucleus and diverge into the post-commissural and pre-commissural fornices.

Projections of the post-commissural fornix which arise from the presubiculum, the parasubiculum, and the subiculum, project to the thalamus, mammillary bodies, and the rostral brain stem (Chronister and DeFrance, 1979). The pre-commissural fornix comprises of fibers from CA1 and CA3 which distribute in the septal nucleus, the diagonal band of Broca, the bed nucleus of the anterior commissure, the lateral preoptic region, and the lateral hypothalamus (Swanson and Cowan, 1977).

6.2.2) Isocortical Projections:

Efferent projections which serve to link the hippocampal formation with the isocortex have been reported between CA1 and various parts of the frontal (Swanson, 1981), the temporal (Schwerdfeger, 1979), the retrosplenial, and the perirhinal cortices (Swanson and Cowan, 1979).
7) ELECTROPHYSIOLOGY OF HIPPOCAMPAL NEURONS:

The RMPs of pyramidal cells range from -50 to -70 mV, whereas that of granule cells range from -60 to -85 mV. Depolarization-evoked action potentials should range from 50 to 110 mV for pyramidal cells and 70 to 140 mV for granule cells on average. By using the slope of a linear portion of an I/V (Current vs Voltage) curve, the average input resistance of granule cells is calculated to be 40 - 45 MΩ. While the average input resistance of CA1 pyramidal cell is about 25 - 30 MΩ, that of CA3 pyramidal cells is approximately 35 MΩ. These values are an order of magnitude higher than the input resistances recorded for spinal cord motorneurons (Barrett and Crill, 1974), but are comparable to those for neocortical neurons (Lux and Pollen, 1966; Connors et al., 1982). The membrane time constants, the time for membrane potential to reach 1-1/e of its peak voltage in response to short current pulses (Spenser and Kandel, 1961a), of the CA3 pyramidal, the CA1 pyramidal, and the granule cells are 25, 15, and 11 ms, respectively. These time constants for hippocampal neurons are much longer than those for motoneurons (Barette and Crill, 1974). Based on electrophysiological data derived from time constant measurements, the ratio of the cell’s dendritic and somatic conductances (ρ) has been reported to be approximately 1.0 - 1.5 (Brown et al., 1981; Johnson, 1981). The small ρ indicates that a large part of the cell is isopotential, including regions that are morphologically considered as dendritic. The electronic length (L) of hippocampal neurons is near 1 (Schwartzkroin and Mueller, 1987). This small L value indicates that current injected in the distal synaptic region would have an appreciable effect on activity initiated at the soma since almost all of the injected current should reach the soma. Recent studies using
the multipolar cylinder model have found the $L$ value to be $1/3$ of the value mentioned previously (Glenn, 1988).
8) IONIC CURRENTS IN HIPPOCAMPAL NEURONS:

8.1) Sodium Currents:

A fast sodium (Na) current (\(I_{Na(fast)}\)) which exhibits the characteristics described by Hodgkin and Huxley (1952) for squid giant axon, namely an activation threshold of -60 mV and a time to peak of about 0.9 ms at 0 mV, exists in hippocampal neurons (Kaneda et al., 1988; Sah et al., 1988a). Inactivation is complete throughout the activation range. The permeability sequence for this current is \(\text{Li} > \text{Na} > \text{hydrazine} > \text{formidime} > \text{guanidine} > \text{methylguanidine} > \text{monomethylamine}\).

This fast Na current is primarily somatic in origin and is responsible for the fast, TTX-sensitive somatic action potentials. QX-312 sensitive Na-spikes which have similar rise times but different decay rates have been reported to exist on isolated dendrites of hippocampus (Benardo et al., 1982). Dendritic Na currents which demonstrate comparable time-course and current density to those in the soma were only observed in freshly dissociated neocortical neurons (Hugenard et al., 1989).

Using the single microelectrode clamp technique, a slowly inactivating Na current, which is sensitive to TTX and QX314 has also been recorded from rat hippocampal CA1 neurons (French and Gage, 1985). The activation threshold of this slow Na current is about 5 to 10 mV positive to the resting membrane potential. Because equivalent rectification in isolated dendrites is insensitive to QX314 and is blocked by Mn\(^{2+}\), the slow Na current may be confined to the soma (Bernado et al., 1982; French et al., 1990). This slow Na current may participate in hippocampal pacemaker activity (Brown et al., 1990) or repetitive firing of action potentials in response to prolonged depolarizations induced by intense synaptic activity (French et al., 1990).
8.2) Calcium Currents:

A high-threshold and sustained (L), a low-threshold (T), as well as a high-threshold and inactivating (N) calcium currents exist in hippocampal neurons (Fox et al., 1987a, b). The conductances of T-, L-, and N-type calcium channels are 7 - 8 pS, 25 - 27 pS, and 13 - 15 pS, respectively (Gray and Johnson, 1987).

8.2.1) High Threshold Sustained (L) Calcium Current:

The L-current is a slowly-activating and sustained inward current which is enhanced by Ba\(^{2+}\) as well as blocked by Co\(^{2+}\) and Cd\(^{2+}\) (Johnson et al., 1980; Brown and Griffith, 1983a; Brown et al., 1984; Docherty and Brown, 1986; Gähwiler and Brown, 1987a). Using Ba\(^{2+}\) as the charge carrier, this current demonstrated little inactivation over several hundred of ms and appeared to be persistent between -50 and -10 mV (Johnson et al., 1980; Brown and Griffith, 1983a). The L-current is sensitive to verapamil (100 µM) (Brown and Griffith, 1983a), ω-conotoxin (µM concentrations) (Mogul and Fox, 1991), and dihydropyridine such as nimodipine (50 nM-10 µM), nifedipine (1 µM), and PY108-068 (1 µM). Bay K8644 (1-10 µM), a calcium agonist, noradrenaline, and isoprenaline have been reported to enhance the L-current (Brown et al., 1984; Docherty and Brown, 1986; Segal and Barker, 1986; Gähwiler and Brown, 1987a; Gray and Johnson, 1987). Activation of the L-channel can be described by the Boltzmann equation with a threshold of approximately -50 mV, half-activation at -20 mV, a slope factor of 10 mV and a cooperativity factor of 2 (Kay and Wong, 1987). The L-current has been suggested to be responsible for hippocampal burst-behavior (Johnson et al., 1980).
8.2.2) Low Threshold Transient (T) Calcium Current:

The low-threshold transient (T) current, which can be activated by a depolarization to about -60 mV from potentials as low as -100 mV and inactivated at -50 mV, has been observed in hippocampal CA1 neurons (Halliwell, 1983; Ozawa et al., 1989; Takahashi et al., 1989). While the T-current is blocked by Cd\(^{2+}\) (100 \(\mu\)M), Ni\(^{2+}\) (IC\(_{50}\) = 0.2 - 0.4 mM), verapamil (100 \(\mu\)M), and phenytoin (Halliwell, 1983; Mogul and Fox, 1991), the T-current is relatively resistant to dihydropyridine (Tsien et al., 1988). By using 10 mM of Ca\(^{2+}\) as the charge carrier and a holding potential of -100 mV, the threshold and the peak of the current were determined to be -60 and -30 mV, respectively. The T-current has been suggested to be involved in the initial phase of burst potentials (Brown and Griffith, 1983a; Traub, 1982), the subthreshold prepotential which confers a Ca\(^{2+}\)-dependent component of inward rectification (Brown and Griffith, 1983a) and pace-maker depolarizations (Llinás and Yarom, 1981).

8.2.3) High Threshold Inactivating Calcium Current:

The N-current which is activated by a depolarization to +10 mV decays over 100 ms (Ozawa et al., 1989; Takahashi et al., 1989b). The N-current inactivates at potentials around -70 mV (Ozawa et al., 1989). The N-type channel, which demonstrates a higher conductance for Ba\(^{2+}\) than for Ca\(^{2+}\) ions (Tsien et al., 1988), is blocked by Cd\(^{2+}\), Ni\(^{2+}\), and \(\omega\)-conotoxin, but is relatively insensitive to dihydropyridine. While activations of adenosine (Madison et al., 1987a) and muscarinic (Gähwiler and Brown, 1987b; Toselli and Lux, 1989) receptors reduce the N-current, noradrenaline and isoprenaline have been reported to augment the current (Gray and Johnson, 1987).
### 8.3) Potassium Currents:

Five different voltage-gated $K^+$ currents, namely a delayed rectifier current $I_{K(DR)}$, a transient or $A$ current $I_{K(A)}$, a delay current $I_{K(D)}$, a subthreshold and non-inactivating current ($M$ current) $I_{K(M)}$, and an inwardly rectifying current $I_{K(IR)}$, are present in hippocampal neurons.

#### 8.3.1) Delayed Rectifier Current $I_{K(DR)}$:

The $I_{K(DR)}$ has been recorded in depolarizing hippocampal neurons at -40 mV (Segal and Braker, 1984; Numann et al., 1987; Sah et al., 1988b). The activation of this current is slow. At 0 mV, the time-to-peak is approximately 50-200 ms. At threshold, the time constant is about 100 ms (Rogawski, 1986). The IC$_{50}$ of TEA for this current is about 10 mM (Segal and Barker, 1984). Even though this current is sensitive to TEA, it is relatively insensitive to 4-AP.

#### 8.3.2) Transient (A) Current $I_{K(A)}$:

This current has been recorded from hippocampal neurons in situ (Gustafsson et al., 1982; Zbicz and Weight, 1985), in culture (Segal et al., 1984; Segal and Barker, 1984), and in acutely dissociated neurons (Numann et al., 1987; Alger and Doerner, 1988; Sah et al., 1988b). The $I_{K(A)}$ activates (time constant = 5-10 ms) and inactivates (time constant = 20-30 ms) rapidly. This current is insensitive to TEA but is readily blocked by 4-AP (>100 μM) and DTX (50-300 nM) (Halliwell et al., 1986). Noradrenaline and acetylcholine have also been reported to inhibit the $I_{K(A)}$ (Sah et al., 1985; Nakajima et al., 1986). This transient potassium current has been suggested to be responsible for spike repolarization in hippocampal cells (Storm, 1987a & b).
8.3.3) Slowly-Inactivating "delay" current $I_{K(D)}$:

Rapid activation is also a characteristic of $I_{K(D)}$. $I_{K(D)}$, which shows multicomponent inactivation over several seconds, has an activation threshold of about -75 mV. This slowly inactivating current is very sensitive to 4-AP (complete block at 30-40 μM) (Storm, 1988) and DTX. In hippocampal cells, $I_{K(D)}$ is believed to introduce a long delay in the firing induced by threshold depolarizations from RMP (Storm, 1988). In other words, firing can only commence when $I_{K(D)}$ is inactivated. Slow inactivation of $I_{K(D)}$ also provides recruitment during repetitive depolarizations.

8.3.4) M-Current $I_{K(M)}$:

This non-inactivating, subthreshold, and voltage-gated K current has been reported in freshly dissected hippocampal slices (Madison et al., 1987b) and cultured neurons (Gähwiler and Brown, 1985a). The current is activated from about -70 mV upwards. At -45 mV and 30 °C, the time constant for activation is about 90 ms (Halliwell and Adams, 1982). Ba$^{2+}$, cholinergic receptor agonists, and serotonin have been reported to inhibit the M current (Colino and Halliwell, 1987; Halliwell and Adams, 1982; Gähwiler and Brown, 1985a; Madison et al., 1987b), whereas somatostatin has been suggested to increase the current (Moore et al., 1988; Watson and Pittman, 1988). The inhibition of $I_{K(M)}$ by acetylcholine is probably caused by inositol 1, 4, 5-triphosphate (Dutar and Nicoll, 1988a).

Although $I_{K(M)}$ does not contribute much to the steady membrane current at the normal RMP (-70 mV), it forms an appreciable fraction of the membrane conductance between -70 and -40 mV. Moreover, this current generates a component of postspike hyperpolarization and contributes to the accommodation which takes place during trains of action potentials (Madison and Nicoll, 1984).
8.3.5) Inwardly-rectifying K current $I_{KIR}$:

$I_{KIR}$ is a rapidly activating (< 10 ms) K current (Owen, 1987). This current partially activates at resting membrane potential (i.e. -60 mV) and inactivates at potentials which are more negative than -100 mV (Owen, 1987). GABA, 5-hydroxytryptamine, and adenosine have been reported to induce an inwardly-rectifying K current in hippocampal neurons (Gähwiler and Brown, 1985b; Newberry and Nicoll, 1985; Colino and Halliwell, 1987; Andrade and Nicoll, 1987). These effects are probably mediated through a pertussis-toxin sensitive GTP-binding protein (Andrade et al., 1986; Zgombik et al., 1989).

8.4) Chloride Current:

A slow Cl⁻ current which was activated by hyperpolarizing steps between -20 and -100 mV has been observed in rat hippocampal slices (Madison et al., 1986). This chloride current is restricted to pyramidal cell processes. The reversal potential for the current was +9.5 mV when measured with microelectrodes filled with Cl⁻ and -71 mV when recorded with microelectrodes filled with MeSO₄ anion. Both Cd²⁺ (Selyanko, 1984) and phorbol dibutyrate (Madison et al., 1986) have been reported to block $I_{Cl}$.

8.5) Current Activated by Hyperpolarization:

When hippocampal cells are hyperpolarized to potentials which are more negative than -80 mV, a mixed cation (Na⁺/K⁺) current called the Q current ($I_Q$) is observed (Adams and Halliwell, 1982; Halliwell and Adams, 1982). Activation of this current is relatively slow. At 30 °C and -82 mV, the time constant of activation is about 100 ms. This time constant increases with increasing hyperpolarization (Halliwell and Adams, 1982). Cs⁺ ions and tetrahydroaminoacridine block the Q
current (Brown et al., 1988). Activation of $I_Q$ serves to resist hyperpolarizing deviations from the resting membrane potential. Deactivation of the current contributes to the rebound depolarization and excitation following a hyperpolarizing current pulse. When action potentials (spikes) are initiated from membrane potentials which are negative to the normal resting potential (Storm, 1989), $I_Q$ may contribute to spike after-hyperpolarization.

8.6) Calcium Activated Currents:

Two potassium currents, a chloride current, and a cation current of hippocampal cells are gated by calcium. These currents are activated by an increase in intracellular calcium produced by calcium entering through voltage-gated calcium channels or through ligand-gated channels (Nicoll and Alger, 1981; Kudo and Ogura, 1986), or by the release of intracellular calcium resulting from activation of muscarinic receptors (Kudo et al., 1988) or metabotropic glutamate receptor (Furuya et al., 1989).

8.6.1) Calcium Activated Potassium currents $I_{K(Ca)}$:

In hippocampal neurons, two calcium activated potassium currents exist. One of these currents is a large, time- and voltage-dependent current ($I_C$) (Brown and Griffith, 1983b), while the other is a smaller and voltage-independent current ($I_{AHP}$) (Lancaster and Adams, 1986).

When a calcium charge flows through voltage-gated calcium channels following their activation by a depolarizing voltage-clamp pulse or during an action potential, $I_C$, which is a large current, is activated rapidly within 1 - 2 ms (Brown and Griffith, 1983b; Lancaster and Adams, 1986; Storm, 1987a; Lancaster et al., 1987). When the cell is repolarized, the current deactivates within 50 - 150 ms (Brown and Griffith, 1983b). This high conductance channel (150 - 270 pS) is blocked by 1 - 10
mM TEA. It requires a high concentration of calcium (> 1 μM) for activation (Franciolini, 1988). This strongly voltage-sensitive current (Franciolini, 1988) contributes to spike repolarization and generates the early phase of the spike after-hyperpolarization (Lancaster et al., 1987; Storm, 1987a, b).

\[ I_{AHP} \], which is smaller than \( I_C \) in amplitude, rises slowly following calcium entry and declines slowly with a time constant of 1 - 1.6 s on repolarization (Lancaster and Adams, 1986). Although the decay rate is insensitive to voltage over the range -50 to -90 mV (Lancaster and Adams, 1986), it is determined by the rate of decline of intracellular calcium (Knöpfel et al., 1989). Activation of \( I_{AHP} \) require a lower intracellular calcium concentration than \( I_C \) (Knöpfel et al., 1989). While \( I_{AHP} \) is insensitive to TEA and charybdo toxin (Lancaster and Adams, 1986; Storm, 1987a; Lancaster et al., 1987), it can be inhibited by acetylcholine (Cole and Nicoll, 1983; Madison et al., 1987b), noradrenaline (Madison and Nicoll, 1982; Haas and Konnerth, 1983; Lancaster and Adams, 1986), histamine (Haas and Konnerth, 1983) and 5-hydroxytryptamine (Andrade et al., 1987; Colino and Halliwell, 1987). Acetylcholine, noradrenaline, histamine, and 5-hydroxytryptamine have been suggested to depress \( I_{AHP} \) by inhibiting the potassium current rather than by blocking the calcium transient (Knöpfel et al., 1989). \( I_{AHP} \) which generates the long after-hyperpolarization following hippocampal action potentials (Lancaster and Adams, 1986; Lancaster et al., 1987), is responsible for the decline in firing frequency, and eventual cessation of firing, during spike trains induced by prolonged depolarization (Madison and Nicoll, 1984). This calcium activated potassium current does not seem to contribute to the resting membrane current.
8.6.2) Calcium Activated Chloride Currents $I_{\text{Cl(Ca)}}$:

A voltage-insensitive calcium activated chloride current ($I_{\text{Cl(Ca)}}$) which has a conductance of 20 pS has been reported in hippocampal cell membrane patches (Owen et al., 1988). This current probably contributes to the long calcium-dependent tail currents (Brown and Griffith, 1983b). In addition, it can generate depolarizing after-potentials in sensory and spinal neurons (Owen et al., 1984; Meyer, 1985). However, whether $I_{\text{Cl(Ca)}}$ contributes to after-potentials in hippocampal cells is not yet clear.

Recent experiments with parallel Fura-2 recording show that a calcium activated cation current probably contributes to an after-depolarization which replaces the normal after-hyperpolarization after $I_{\text{AHP}}$ is suppressed with muscarinic agonists (Benardo and Prince, 1982; Gähwiler, 1984; Knöpfel et al., 1989).

8.7) Miscellaneous Membrane Currents:

8.7.1) Leak Currents:

The currents which remain around the resting membrane potential when voltage- and calcium-gated currents are suppressed are referred as leak currents. One component of this residual conductance is a voltage-insensitive potassium conductance which can be reduced by muscarinic agonists (Madison et al., 1987b; Benson et al., 1988). Several types of chloride channels have also been suggested as a component of this leak currents (Franciolini and Nonner, 1987; Franciolini and Petris, 1988; Owen et al., 1988).

8.7.2) Sodium Activated Current:

The prolonged after-hyperpolarization induced by tetanic stimulation of CA3 neurons is probably caused by electrogenic sodium extrusion by the sodium pump
and a sodium activated potassium current (Gustafsson and Wigström, 1983). The
significance of this post-tetanic sodium activated potassium current to hippocampal
cell behavior is unclear.

8.7.3) ATP-Gated Potassium Channel:

During anoxia, the potassium conductance of rat hippocampal CA1 neurons is
increased (Hansen et al., 1982). Because hyperpolarization induced by the increase
in potassium conductance during anoxia is blocked by sulphonarylurea compounds, this
potassium conductance may be an ATP-blocked potassium channel. This ATP-
regulated potassium channel normally does not contribute to the resting membrane
potential under normal situations since sulphonarylurea does not alter the resting
membrane potential or conductance in the absence of anoxia (Krnjevic and Leblond,
1988; Mourre et al., 1989).
9) FIELD POTENTIALS IN THE HIPPOCAMPUS:

Granule cells receive excitatory afferents from entorhinal cortex via the perforant pathway (Andersen et al., 1966a, b; Lomo, 1971). Perforant path fibers from lateral entorhinal cortex produce EPSPs on the middle third of the dendritic tree (Blackstad, 1958; Hjorth-Simonsen and Juene, 1972; Steward, 1976; McNaughton and Barnes, 1977). The rate of rise for more distal EPSPs is slower than that for the medial entorhinal EPSPs (Abraham, 1982). Like perforant path fibers, commissural afferents from contralateral pyramidal cells (Blackstad, 1956), associational fibers from CA3 and CA4 neurons (Zimmer, 1971), and septal fibers (Rose et al., 1976), which synapse on proximal dendrites, also produce excitatory effects (Steward et al., 1977; Fantie and Goddard, 1982).

Mossy fibers from the granule cells in the dentate gyrus project a sufficiently large excitatory input to the proximal dendrites of CA3 pyramidal neurons, whereas CA1 neurons receive their excitatory inputs from Schaffer collaterals. Besides mossy fibers, commissural, entorhinal, and septal inputs also contribute to excitatory inputs to CA1 and CA3 cells (Andersen and Lomo, 1966; Andersen et al., 1966, 1971; Stanley et al., 1979).

9.1) EPSPs and IPSPs:

EPSPs produced by stimulation of the distal portions of the pyramidal and granule cell dendrites have slower rise times than those generated at more proximal locations. The discrepancy between the rise times of the proximally and distally generated EPSPs has been attributed to electronic decay which occurs as the EPSP travels from the distal dendrites to the soma. The different characteristics of the
inputs synapsing with the distal dendritic portions are also responsible for the variable rise times (Langmoen and Andersen, 1981; Andersen et al., 1980; Abraham, 1982).

Excitatory afferents which synapse on pyramidal cell dendrites may be amplified by the intrinsic dendritic mechanisms such as dendritic spikes and dendritic calcium depolarizations (Hamlyn, 1963; Andersen et al., 1966). The EPSP produced by the afferent volley is followed by a large IPSP which limits the excitatory response to a single action potential (Kandel et al., 1961; Dunwiddie et al., 1980). This inhibition is probably mediated by inhibitory interneurons which demonstrate features of basket cells (Andersen et al., 1964a,b, 1969; Schwartzkroin and Mathers, 1978; Lee et al., 1980; Fox and Ranck, 1981). These basket cell-like interneurons are located basal to the pyramidal cell somata and are excited by axon collaterals of pyramidal neurons (Knöwles and Schwartzkroin, 1981).

Orthodromic stimulation of the stratum radiatum has been reported to produce an early chloride-mediated IPSP and a later, slow hyperpolarization through activation of a feed-forward inhibitory system (Fujita, 1979; Thalmann and Ayala, 1982; Alger and Nicoll, 1982; Knowles et al., 1982; Alger, 1984). While the fast component is mediated by an increase in chloride conductance which has a reversal potential of approximately -70 mV (Spencer and Kandel, 1961a; Allen et al., 1977; Eccles et al., 1977; Dingledine and Langmoen, 1980; Alger and Nicoll, 1982), the slower component is attributed to an increase in potassium conductance (Alger, 1984). Antidromic stimulation of alvear fibers has also been reported to produce IPSPs in pyramidal cells through activation of a recurrent inhibitory circuit (Andersen et al., 1964b; Kandel et al., 1961). While orthodromic stimulations of the stratum radiatum elicits both fast and slow IPSPs (Alger and Nicoll, 1982; Fujita, 1979), antidromic
stimulations of the alveus predominantly produce fast IPSPs (Alger and Nicoll, 1982; Andersen et al., 1964b; Dingledine and Langmoen, 1980). This observation suggests that the fast IPSP is generated near the pyramidal cell soma and dendrites and the slow IPSP is elicited at the dendrites.

9.2) Population Synchronization:

Both the afferent EPSP pathways and the local IPSP circuits may potentially synchronize in large populations of pyramidal cells. When many neurons are synchronously activated, stimulation of an afferent input can generate the population or field EPSP, which is characterized by a large negative wave when recording at the dendrites (sink) and a positive deflection when recording at the somata (source). If the synaptic excitation exceeds the threshold for spike generation, the field EPSP will be interrupted by a population spike which is a negative wave when recorded near the cell body layer and a positive wave when recorded near the dendrites. The number of synchronously discharging neurons can be estimated by the amplitude and width of the population spike (Andersen et al., 1971a). At a fixed stimulation strength, the amplitudes of the field EPSP and the population spike vary according to the distance between the source and the recording electrode.
10) EXCITATORY AMINO ACID RECEPTORS IN THE HIPPOCAMPUS:

Excitatory amino acid receptors can be classified into five different types. They are the NMDA, the AMPA, the kainate, the L-AP4, and the metabotropic (Glu₆) receptors. In this section, the properties of these receptors will be reviewed briefly.

10.1) The NMDA Receptor:

The NMDA receptor channel is voltage-dependent (MacDonald & Porietis, 1982; MacDonald et al., 1982), permeable to $K^+$, $Na^+$, and $Ca^{2+}$ ions (MacDermott et al., 1986; Mayer and Westbrook, 1987b; Ascher and Nowak, 1988b), and blocked by $Mg^{2+}$ in a voltage-dependent manner (Nowak et al., 1984; Mayer and Westbrook, 1987b; Jahr and Stevens, 1990). This receptor has a single channel conductance between 40 and 50 pS. Over a holding potential range of -100 to +30 mV, the current-voltage (I-V) relationship recorded from neurons exposed to NMDA is biphasic (Mayer et al., 1984). This biphasic I-V relationship has been suggested to be due to the voltage-dependent and uncompetitive blockade of the NMDA receptor channel by $Mg^{2+}$ at negative potentials (Mayer et al., 1984; Mayer and Westbrook, 1987b; Nowak et al., 1984). While maximum inward current through the NMDA receptor channel occurs at about -30 mV, the current reverses in direction at 0 mV. NMDA receptor-mediated synaptic currents demonstrate slow onset (8 - 20 ms) and long duration (60 - 150 ms) (Collingridge et al., 1988; Forsythe and Westbrook, 1988; Hestrin et al., 1990). The long time course of the NMDA receptor evoked current has been suggested to be the result of prolonged occupation of the receptor by glutamate (Lester et al., 1990).

Compounds such as APV, PCP, ketamine, and MK 801 have been reported to block NMDA receptor-mediated responses. While APV competitively blocks the
NMDA receptor (Davies et al., 1981; Evans et al., 1982), PCP, ketamine, and MK 801 block the channel in a highly voltage- and use-dependent manner (Hicks and Guedes, 1981; MacDonald et al., 1987).

NMDA binding is dense in the outer two-thirds of the molecular layer of the dentate gyrus, and in the stratum oriens and the stratum radiatum of CA1 and CA3. While NMDA binding is absent in the stratum lucidum of CA3, NMDA binding is dense in the stratum radiatum of CA1. In the subiculum, weak NMDA binding is also present.

Entry of Ca\(^{2+}\) through the NMDA receptor channel has been shown to underlie LTP (Kelso et al., 1986; Malenka et al., 1988) and excitotoxic degeneration in cultured neurons (Garthwaite et al., 1986; Choi, 1987; Abele et al., 1990; Michaels and Rothman, 1990).

10.1.1) Transmitter Recognition Domains:

Binding sites for agonists and antagonists on the NMDA receptor are not identical (Watkins and Olverman, 1988; Fagg and Baud, 1988; Olverman and Watkins, 1989). While the 1-carboxyl and \(\alpha\)-amino groups of agonists and antagonists interact with the same charged residues on the receptor surface, the \(\omega\)-acidic terminals of agonists and antagonists bind to different sites on the receptor.
10.1.2) Allosteric Modulation by Glycine:

By using patch clamp techniques in cultured neurons, submicromolar concentrations of glycine have been reported to increase the frequency of NMDA-induced channel opening (Johnson and Ascher, 1987). Because this effect of glycine is insensitive to strychnine, it is not likely to be caused by the inhibitory actions of glycine in the brainstem and spinal cord. For this reason, glycine may act as an agonist at an allosteric regulatory site on the NMDA receptor.

Glycine has been reported to reduce desensitization of the NMDA current by increasing the rate constant of recovery from desensitization (Mayer et al., 1989). In addition, glycine site agonists or partial agonists (glycine, D-serine, D-cycloserine) have been shown to enhance seizure activity (Larson and Beitz, 1988; Singh et al., 1990a), to reverse the effects of PCP or MK 801 (Toth and Lajitha, 1986; Contreras, 1990), and to enhance learning performance (Monahan et al., 1990).

10.1.3) PCP Channel Binding Site:

MK 801, a highly potent and selective NMDA receptor antagonist of the PCP-type (Wong et al., 1986), blocks NMDA receptor responses in an uncompetitive, use-dependent, and voltage-dependent manner by binding inside the open channel and blocking transmembrane ion fluxes (Honey et al., 1985; Huettner and Bean, 1988; MacDonald et al., 1987; Martin and Lodge, 1985). Based on radioligand binding studies, PCP and related substances do not interact with the transmitter recognition site.
10.1.4) Modulation by Polyamine:

Polyamines have been reported to increase the level of binding of glutamate and glycine. While spermidine has been shown to enhance NMDA-induced whole-cell currents in cultured neurons (Sprosen and Woodruff, 1990), arcaine has been reported to block the NMDA-evoked release of $[^{3}\text{H}]$noradrenaline from brain slices (Sacaan and Johnson, 1990). In vivo, spermidine has been observed to potentiate NMDA-induced seizures (Singh et al., 1990b). Ifenprodil which shows neuroprotective properties in vivo (Gotti et al., 1988) has been suggested to interact with the polyamine site on the NMDA receptor (Carter et al., 1989; Schoemaker et al., 1990).

10.1.5) Zn$^{2+}$ Binding Site:

Low concentrations ($\mu$M) of Zn$^{2+}$ have been reported to block NMDA receptor responses (Westbrook and Mayer, 1987; Peters et al., 1987). Unlike the Mg$^{2+}$ blockade, Zn$^{2+}$ blockade is voltage-independent (Westbrook and Mayer, 1987). Zn$^{2+}$ has also been suggested to modulate the binding kinetics of $[^{3}\text{H}]$MK 801 (Reynolds and Miller, 1988) and to non-competitively inhibit the binding of $[^{3}\text{H}]$glycine (Yeh et al., 1990). Because Zn$^{2+}$ is localized within and released from synaptic terminals during excitatory activity (Crawford and Connor, 1972; Assaf and Chung, 1984), it may play a regulatory role in NMDA receptor-mediated events in some regions of the CNS (Weiss et al., 1989).

10.2) Non-NMDA Receptors:

As a result of the voltage-dependent blockade by Mg$^{2+}$, the participation of NMDA receptors in mediating the EPSP induced by a unitary stimulation is limited. While the NMDA receptor is responsible for the voltage-dependent component of the
EPSP, the voltage-independent portion of the EPSP is attributed to the kainate and AMPA receptors.

10.2.1) The AMPA Receptor:

AMPA receptors are distributed extensively in the cortex, hippocampus, lateral septum, striatum, and the molecular layer of the cerebellum (Monaghan et al., 1984; Nielsen et al., 1988, 1990). Because the distribution of AMPA receptors corresponds closely to that of NMDA receptors, these two receptor subtypes may act in concert to activate postsynaptic neurons. The conductance of the AMPA receptor channel is intermediate in size between those of NMDA and kainate receptor channels (5 - 15 pS) (Ascher and Nowak, 1988a; Cull-Candy et al., 1988). The reversal potential for AMPA receptor channels is 0 mV. These channels which show little voltage-dependence are permeable to Na\(^+\) and K\(^+\) (Mayer and Wesbrook, 1987a, b; Ascher and Nowak, 1988a).

Although both AMPA and quisqualate activate the AMPA receptors, AMPA binds the receptors with much higher selectivity than quisqualate (Krogsgaard-Larsen et al., 1980). Compounds such as CNQX, NBQX, and barbiturates have been reported as antagonists of AMPA receptors (Honore et al., 1988; Sheardown et al., 1990). On the other hand, Zn\(^{2+}\)(Koh and Choi, 1988; Rassendren et al., 1990) and aniracetam agents (Ito et al., 1990) have been suggested to potentiate AMPA receptor responses.

AMPA binding is more prominent in the outer two-thirds of the molecular layer of dentate gyrus. In the stratum oriens and the stratum radiatum of both CA1 and CA3, AMPA binding is also dense. In comparison to CA1, AMPA binding in the subiculum is less.
10.2.2) The Kainate Receptors:

Neurophysiological actions of kainate have been suggested to be mediated via the AMPA receptor (Watkins et al., 1990). In addition, cloning of the kainate binding site has implied that the kainate receptor is equivalent to the AMPA receptor (Gregor et al., 1989; Hollmann et al., 1989; Wada et al., 1989). However, neurotoxicity data suggest that the pharmacology of kainate-induced excitotoxicity is not the same as that of the AMPA receptor (Coyle et al., 1984). Electrophysiological studies have identified a group of C-fiber afferents to the spinal cord which are sensitive to kainate but not to AMPA. In cultured neurons, kainate and AMPA activate channels which demonstrate different electrophysiological properties. Binding studies have indicated a high affinity \(^{3}H\)kainate binding site which distributes differently from the AMPA receptor (Monaghan and Cotman, 1982; Young and Fagg, 1990). In addition, the regional distribution of this high affinity \(^{3}H\)kainate binding site is similar to the distribution of kainate induced neurotoxicity (Perl et al., 1990; Teitelbaum et al., 1990).

The \(K_D\) of the kainate binding site is in the nM range and is blocked by kainate analogues (domoate > kainate > quisqualate > glutamate), \(Ca^{2+}\), CNQX, and DNQX. Kainate binding is most intense in the stratum lucidum of CA3. A thin band of dense binding is present in the inner third of the molecular layer of dentate gyrus and in CA4. While kainate binding in CA1 is virtually absent, moderate binding exists in the subiculum.

10.2.3) The L-AP4 Receptor:

L-AP4 receptor was identified on the basis of the potent antagonistic properties of L-AP4 at excitatory synapses (\(K_D = 2.5 \mu M\)) (Koerner and Cotman, 1981; Collins,
While electrophysiological studies have shown that L-AP4 blocks excitatory synaptic responses at low micromolar concentrations, L-AP4 does not block the depolarizing actions of glutamate, NMDA, quisqualate, and kainate (Hori et al., 1981; Ganong and Cotman, 1982). L-AP4 receptor has been suggested to be a presynaptic autoreceptor which inhibits transmitter release (Collingridge et al., 1984b; Anson and Collins, 1987).

10.2.4) The Glu<sub>G</sub> Receptor:

In cultured mouse striatal neurons, quisqualate and L-glutamate were observed to stimulate phosphoinositol (PI) turnover via a pertussis toxin-sensitive G-protein with EC<sub>50</sub>'s of 0.16 and 4 μM, respectively (Sladeczek et al., 1985). Unlike quisqualate and L-glutamate, NMDA and kainate were less potent in stimulating the PI turnover. Not only are these observations reported in mouse striatal neurons, but also in cultured neurons from several different regions of the brain, cultured astrocytes, hippocampal slices, and synaptoneurosomes (Nicoletti et al., 1986b; Récasens et al., 1987; Schoepp and Johnson, 1988; Palmer et al., 1988; Ambrosini and Meldolesi, 1989; Patel et al., 1990; Pearce et al., 1990). The metabotropic receptor is most abundant in the dentate gyrus and CA3. Binding in CA1 is considerably less than in CA3 and the dentate gyrus.

In comparison to quisqualate, ibotenate, and L-glutamate, trans-ACPD has been shown to increase the PI turnover in hippocampal slices with a higher selectivity (Palmer et al., 1989; Watson et al., 1990). Antagonists such as AP3 and AP4 have been reported to exhibit weak antagonistic property at the Glu<sub>G</sub> receptor (Schoepp and Johnson, 1988, 1989).
Glu₆ receptors have been reported to be involved in synaptic plasticity (Sladeczek et al., 1988; Monaghan et al., 1989). The activity of the receptor declines during CNS maturation (Nicoletti et al., 1986a) and reappears following deafferentation, brain ischemia or kindling (ladorola et al., 1986; Nicoletti et al., 1987; Akiyama et al., 1989; Seren et al., 1989). Activation of the Glu₆ receptor also blocks the slow afterhyperpolarization and accommodation of firing in CA1 neurons following depolarizing current injections (Stratton et al., 1990).
11) GABA-ERGIC SYNAPTIC TRANSMISSION:

GABA-ergic markers such as GAD which is the GABA synthetic enzyme (Ribak et al., 1978; Somogyi et al., 1983), GABA-T which is the GABA inactivating enzyme (Nagai et al., 1983), and GABA itself (Storm-Mathisen et al., 1983), are present in all hippocampal laminae. Around the somata of pyramidal cells in CA1-CA3 regions and around granule cells in the fascia dentata, a dense plexus formed by GABA-ergic terminals and fibers has been reported in the literature (Ribak et al., 1978; Storm-Mathisen et al., 1983; Somogyi et al., 1983; Misgeld and Frotscher, 1986; Woodson et al., 1989). Not only are GABA-ergic boutons found in the somata of pyramidal and granule cells, but also on their axon hillock (Somogyi et al., 1983; Soriano and Frotscher, 1989), as well as the basal and apical dendrites (Somogyi et al., 1983; Woodson et al., 1989). While the density of GABA-ergic synapses is fairly homogenous in dendritic fields of CA1-CA3 area (Woodson et al., 1989), the density of GABA-ergic synapses is highest in the outer third of the molecular layer of the fascia dentata (Woodson et al., 1989). In the dendritic layers of both CA1-CA3 areas and fascia dentata, GABAergic boutons are also present on the somata and processes of hippocampal GABA-ergic interneurons (Misgeld and Frotscher, 1986; Freund and Antal, 1988; Woodson et al., 1989).

Using autoradiographic studies, moderate levels of GABA receptor have been observed in all layers of CA1-CA4 regions and dentate gyrus (Bowery et al., 1987). Unlike GABA receptor, GABA receptor are distributed in a less homogenous manner. The densities of GABA receptor in the CA1-CA4 pyramidal layer and the granular layer of the fascia dentata are lower than that in the dendritic layers of these regions.
GABA-ergic cells, which constitute 11% of the total population of hippocampal neurons (Woodson et al., 1989), are heterogenous in morphology. They include basket, stellate, and horizontal cells which are short axon neurons (Ribak et al., 1978; Seress and Ribak, 1983; Nagai et al., 1983; Woodson et al., 1989). Somata of most GABA-ergic neurons are located outside the pyramidal layers of CA areas and the granule cell layer of fascia dentata, and predominantly in the CA1-CA3 dendritic regions. (Woodson et al., 1989).

The hippocampal formation receives GABA-ergic afferents from the entorhinal cortex via the perforant path (Germroth et al., 1989), and from the septum via the fimbria-fornix (Köhler et al., 1984; Freund and Antal, 1988). Most GABA-ergic hippocampal interneurons receive input from GABA-ergic septohippocampal fibers (Freund and Antal, 1988).

11.1) Spontaneous IPSPs:

Spontaneous IPSPs were observed in rat hippocampal slices by Alger and Nicoll (1980a). By using microelectrodes filled with potassium acetate, hyperpolarizing spontaneous IPSPs were recorded from guinea pig CA3 pyramidal cells (Miles and Wong, 1984).

Spontaneous IPSPs are GABAergic in nature because they are sensitive to bicuculline (1-100 μM) (Alger and Nicoll, 1980a; Collingridge et al., 1984a) and picrotoxinin (100 μM) (Miles and Wong, 1984). In addition to bicuculline and picrotoxinin, spontaneous IPSPs are sensitive to pentobarbitone, flurazepam, d-tubocurarine, and folic acid. Pentobarbitone (50-100 μM) has been reported to enhance the duration and amplitude of the spontaneous IPSPs (Alger and Nicoll, 1980a; Collingridge et al., 1984a). In mouse dentate granule cells, ionophoresis of
flurazepam was also reported to enhance the amplitude of spontaneous IPSPs (Biscoe and Duchen, 1985a). Both d-tubocurarine (10-50 μM) and folic acid (0.1-1 mM) have been observed to block spontaneous IPSPs in guinea pig CA3 neurons and rat CA1 neurons, respectively, via a postsynaptic mechanism (Lebeda et al., 1982; Otis et al., 1985).

11.2) Characteristics of IPSPs Evoked in CA1-CA3 Regions:

Chloride-sensitive inhibitory synaptic potentials evoked by fornix stimulation were reported in cat CA2-CA3 pyramidal cells by Kandel et al. (1961). Stimulation of commissural, septal, and fimbrial pathways can also elicit hyperpolarizing synaptic potentials (Andersen et al., 1963, 1964a,b). Activation of interneurons which synapse on the somata of pyramidal cells, such as basket cells, has been suggested to mediate hippocampal inhibition induced by stimulating the above pathways (Andersen et al., 1963, 1964a,b).

11.3) Antidromic or Feed-back IPSPs:

Stimulation of axons of hippocampal pyramidal cells such as the alveus and the Schaffer collaterals (i.e. antidromic stimulation) has been reported to evoke a monophasic fast hyperpolarizing potential in the cellular field of origin (Kandel et al., 1961; Dingledine and Langmoen, 1980; Biscoe and Duchen, 1985b). This fast IPSP which is associated with a decrease in the input resistance of the pyramidal cell (Dingledine and Langmoen, 1980) has a reversal potential of -65 to -75 mV when measured with potassium acetate microelectrodes (Andersen et al., 1980; Alger and Nicoll, 1982; Biscoe and Duchen, 1985b). Because the amplitude of the IPSP decreases after extracellular chloride ions are replaced with ethionate (Alger and Nicoll, 1982) and intracellular injections of Cl\(^-\) reverse the antidromic IPSP (Kandel et
An increase in the membrane permeability to Cl⁻ has been suggested to underlie the generation of the fast IPSP. In addition to its chloride sensitivity, the fast IPSP can be blocked by ionophoresis of bicuculline methiodide (Alger and Nicoll, 1982) and by superfusion with picrotoxinin (1-100 µM) (Alger, 1984; Miles and Wong, 1984). These observations suggest the involvement of GABA_{A} receptors in the production of the fast IPSP.

The antidromic IPSP is generated by a feed-back circuit which is formed by recurrent collaterals of the efferent axons of pyramidal cells that synapse onto inhibitory interneurons (MacVicar and Dudek, 1980; Knowles and Schwartzkroin, 1981; Miles and Wong, 1984). Even though basket cells predominantly provide GABAergic synaptic contacts onto the somata of pyramidal cells, axo-axonic GABAergic cells which synapse onto the axon initial segment may also contribute to feed-back or antidromic IPSPs since recurrent IPSPs have been reported in the absence of axosomatic synapses (Somogyi et al., 1983).

11.4) **Orthodromic or Feed-forward IPSPs:**

Unlike antidromic stimulation, orthodromic stimulation elicits an EPSP and a biphasic IPSP (Alger and Nicoll, 1982; Newberry and Nicoll, 1984; Alger, 1984; Knowles et al., 1984; Biscoe and Duchen, 1985b). In addition to the synaptic potentials, an even slower hyperpolarization which is probably due to the activation of a Ca^{2+}-dependent K⁺ current can be observed when the EPSP produces an action potential (Alger and Nicoll, 1980b; Hotson and Prince, 1980; Newberry and Nicoll, 1984).

Like the fast IPSP generated by antidromic stimulation, the orthodromic stimulation-induced fast IPSP is also associated with a decrease in the input
resistance which is caused by an increase in Cl⁻ conductance (Knowles et al., 1984). GABA\(_A\) receptors are also involved in the generation of the orthodromic stimulation-induced fast IPSP because superfusion with bicuculline (1-10 \(\mu\)M) or picrotoxinin (1-10 \(\mu\)M) has been reported to remove the fast IPSP (Knowles et al., 1984). The reversal potential of the fast IPSP is normally 10-20 mV more negative than the resting membrane potential and is identical to the reversal potential for GABA (Ben-Ari et al., 1981; Knowles et al., 1984; Biscoe and Duchen, 1985b; Misgeld et al., 1986).

The fast IPSPs appear to be generated predominantly on the dendrites of the pyramidal cells since they are very sensitive to dendritic ionophoresis of bicuculline methiodide (Alger and Nicoll, 1982). Depending on the proportion of pyramidal cells that reach the firing threshold at any given intensity of an orthodromic stimulation, the orthodromic fast IPSP may contain somatic components elicited by recurrent collateral fibers. In other words, the fast IPSP may be generated by inhibitory synapses which are located on the pyramidal cell soma (Sivilotti and Nistri, 1991).

In the presence of barbiturates, the hyperpolarizing fast IPSP can be transformed to a biphasic hyperpolarizing-depolarizing response. This depolarizing component of the fast IPSP, which can be enhanced by GABA uptake inhibitors, is generated in the dendrites by GABA\(_A\) receptor activation since it can be inhibited by dendritic ionophoresis of bicuculline methiodide or TTX (Alger and Nicoll, 1982). Normally, the depolarizing component is not detected because GABA uptake limits the diffusion of GABA from the synapses (Avoli and Perreault, 1987). The ionic mechanisms underlying the depolarizing component of the orthodromic fast IPSPs have been suggested to involve either the activation of a mixed Cl⁻/cation
conductance or the activation of a Cl⁻ conductance in parts of the dendritic tree which has an outward electrochemical gradient for Cl⁻ (Silvilotti and Nistri, 1991).

Unlike the fast IPSP, the slow IPSP appears at higher intensities of stimulation (Newberry and Nicoll, 1984), has a slower time course which lasts for hundreds of milliseconds (Newberry and Nicoll, 1984; Alger, 1984; Hablitz and Thalmann, 1987), and is associated with a modest increase in conductance (Knowles et al., 1984; Alger, 1984). The reversal potential of the slow IPSP, which is sensitive to the extracellular K⁺ concentration has been estimated to be about -95 mV (Hablitz and Thalmann, 1987). The slow IPSP is not sensitive to changes in intracellular and extracellular Cl⁻ concentrations (Knowles et al., 1984; Newberry and Nicoll, 1984; Biscoe and Duchen, 1985c) and is enhanced rather than being blocked by GABAₐ antagonists (Newberry and Nicoll, 1984). The enhancement of the slow IPSP by GABAₐ antagonists could be due to removal of GABAₐ-mediated inhibition of GABAergic interneurons (Newberry and Nicoll, 1984).

Both phaclofen (0.2-0.5 mM) (Dutar and Nicoll, 1988b; Soltesz et al., 1988) and 2-hydroxysaclofen (50-200 μM) (Lambert et al., 1989) have been reported to block the slow IPSP. Pretreatment with pertussis toxin (Dutar and Nicoll, 1988c) or intracellular injection of GTP-γ-S (Thalmann, 1988), which blocks the coupling of GABA₉ receptors to K⁺ channels, can also abolish the slow IPSP.

In general, the orthodromic IPSP is mediated by a direct activation of afferent fibers of GABAergic interneurons which synapse predominantly onto the dendrites of pyramidal cells (i.e. feed-forward inhibition) (Alger and Nicoll, 1982). A somatic inhibitory component which is induced by feed-forward mechanisms or by the stimulation of feed back circuits brought about by the activation of the pyramidal cells
may also contribute to the mediation of the slow IPSP. In terms of recurrent inhibition, it is difficult to ascribe feed-forward IPSPs to the activity of a single set of interneurons.

11.5) IPSPs of Granule cells:

In hippocampal granule cells, inhibitory synaptic responses include a GABA_A mediated fast IPSP and a GABA_B mediated slow IPSP have been reported. The fast IPSPs which are Cl^-dependent have been recorded following both orthodromic and antidromic stimulations (Thalmann and Ayala, 1982; Misgeld et al., 1986). Using microelectrodes filled with potassium acetate and sulfate, the reversal potentials for the GABA_A mediated fast IPSP is -75 and -62 mV, respectively. Compounds such as picrotoxinin (50 μM) (Thalmann and Ayala, 1982), penicillin, bicuculline, and pentylenetetrazol (Fricke and Prince, 1984) have been observed to block the fast IPSP.

Like the fast IPSP, the slow IPSP, which is sensitive to changes in extracellular levels of K^+ and picrotoxinin, has been observed in response to both antidromic and orthodromic granule cell stimulation. The reversal potential of this slow IPSP has been estimated to range from -80 to -90 mV (Thalmann and Ayala, 1982; Biscoe and Duchen, 1985b).

Even though dentate basket cells have been suggested to play a major role in granule cell inhibition (Andersen et al., 1966), a GABAergic axo-axonic interneuron which may mediate the inhibition elicited by perforant path stimulation may also be involved (Soriano and Frotscher, 1989). While the dendrites of the axo-axonic interneuron are mainly in the molecular layer where perforant path fibers terminate, their axons synapse on the axon of the initial segment of granule cells. In general,
both feed-forward and feed-back circuits contribute to granule cell inhibition (Buzsáki, 1984).

11.6) Interneurons and Inhibitory Synaptic Transmission:

Hippocampal interneurons are large somata whose sizes range from 35 to 50 µm on average. They are characterized by their aspinous dendrites and locally arborizing axons (Ribak and Andersen, 1980). In this section, interneurons located in stratum pyramidale, near the border between the oriens and the alveus, as well as near the border between the stratum radiatum and lacunosum-molecular, which are involved in inhibitory synaptic transmission will be discussed.

11.6.1) Basket Cells:

Basket cells, whose axonal plexus resembles a basket around the target somata (Cajal, 1911; Lorente de Nó, 1934), are present in both strata pyramidale and granulosum. The somata of baskets cells have an average size of 45 µm. The aspinous dendrites of baskets cells, which show periodic swellings, receive multiple synaptic contacts. Basket cells have been reported to exert feedback (recurrent) inhibitions on pyramidal neurons (Kandel et al., 1961; Andersen et al., 1964). Even though basket cells are thought to be involved in the feed-back inhibition, basket cells may also take part in the feed-forward inhibition since stimulations of the Schaffer collateral/commissural afferents can activate basket cells (Alger and Nicoll, 1982; Ashwood et al., 1984; Buzsaki and Eidelberg, 1982).

Because these interneurons are immunoreactive for GABA (Gamrani et al., 1986) and GAD (Ribak et al., 1978), GABA may be involved in the basket cell-mediated inhibition. The membrane time constants of basket cells are approximately 3 ms. Brief action potentials (0.8 ms) associated with large after hyperpolarizing
potentials (5-10 mV) and tonic depolarization-induced non-accommodating spike discharges are both characteristics of basket cells.

11.6.2) Interneurons at the border between the Oriens and the Alveus:

The oriens/alveus interneurons, which are multipolar and 20 to 30μm in diameter, have been reported to display both GABA-like (Gamrani et al., 1986) and somatostatin-like immunoreactivity (Kohler and Chan-Palay, 1982; Morrison et al., 1986). Aspinous dendrites with periodic swellings are also characteristics of these interneurons. While most dendrites of oriens/alveus interneurons are arranged parallel to the alveus, some turn and project into the stratum oriens, the stratum pyramidale, the stratum radiatum, and the stratum lacunosum-moleculare. The axons of oriens/alveus interneurons are distributed in the stratum oriens and the stratum pyramidale.

This type of interneuron has a short membrane time constant of 6 ms. Like basket cells, oriens/alveus interneurons also produce brief action potentials associated with large after-hyperpolarizations and tonic depolarization-induced non-accommodating spike discharge. These interneurons are involved in both feed-forward and feed-backward inhibitions since they can be activated either by stimulation of the Schaffer collateral/commissural afferents or by depolarizing pyramidal cells which are synaptically paired with the interneurons.

11.6.3) Lacunosum-moleculare Interneurons:

Lacunosum-moleculare interneurons, which are characterized by fusiform or multipolar somata, are GABAergic inhibitory interneurons. These interneurons are located at the border between strata lacunosum-moleculare and radiatum (Kawaguchi and Hama, 1987; Lacaille and Schwartzkroin, 1988). Dendrites of lacunosum-
molecular interneurons run parallel to stratum lacunosum-moleculare and project into the stratum pyramidale and the stratum oriens of the hippocampus proper, as well as the stratum moleculare of the dentate gyrus. Axons of these interneurons also project in a similar fashion (Kunkel et al., 1988; Lacaille and Schwartzkroin, 1988). Because lacunosum-moleculare interneurons are neither excited nor inhibited by pyramidal cells which are synaptically synapse with them, these interneurons have been suggested to mediate feed-forward inhibition. (Lacaille and Schwartzkroin, 1988).

Like basket and oriens/alveus interneurons, lacunosum-moleculare interneurons also demonstrate spike after-hyperpolarizations and little spike accommodation to depolarizing current injections. The action potential duration and the membrane time constant of the lacunosum-moleculare interneuron are 2 and 9 ms, respectively.

11.7) Presynaptic GABA Receptors:

Baclofen, a GABA analogue which does not interact with muscimol- and bicuculline-sensitive GABA receptors, and GABA have been reported to inhibit release of noradrenaline (Bowery and Hudson, 1979; Bowery et al., 1981), acetylcholine (Brown and Higgins, 1979) from peripheral nerve endings, and that of noradrenaline (Bowery et al., 1980), dopamine (Bowery et al., 1980; Reimann et al., 1982), serotonin (Bowery et al., 1980; Schlicker et al., 1984; Gray and Green, 1987), and glutamate (Potashner, 1979) in the central nervous system. Similarly, the release of \[^{3}H\]GABA elicited by 15 mM K\(^+\) from synaptosomes of the median eminence was also inhibited by baclofen (Andersen and Mitchell, 1985). Based on this observation, Andersen and Mitchell first implicated the existence of GABA\(_B\) autoreceptors in the control of GABA release in 1985.
In addition to the above observations, electrophysiological data also support the existence of the GABA<sub>B</sub> autoreceptor. Baclofen has been reported to attenuate paired-pulse inhibition of hippocampal CA1 pyramidal neurons induced by stimulation of Schaffer collaterals, which is mediated by postsynaptic GABA<sub>A</sub> receptors, at concentrations which do not produce postsynaptic effects on pyramidal cells (Karisson and Olpe, 1989). For this reason, baclofen was suggested to reduce paired-pulse inhibition via reduction of GABA release from interneurons (Karisson and Olpe, 1989). Moreover, both phaclofen and CGP 35348, GABA<sub>B</sub> antagonists, have been reported to attenuate the effect of baclofen on paired-pulse inhibition (Pozza et al., 1989).

Besides the reduction of paired-pulse inhibition, IPSPs elicited by orthodromic stimulations are also reduced by baclofen. Because this effect outlast the postsynaptic effects of baclofen, the attenuation of IPSPs is probably mediated by a reduction of GABA release (Deisz and Prince, 1989; Deisz and Ziegglänsberger, 1989).

Although the existence of GABA<sub>B</sub> autoreceptors in synaptosomes of the rat median eminence, slices of rat hippocampus, and slices of the striatum has been affirmed (Andersen and Mitchell, 1985; Waldmeier et al., 1988), the existence of such an autoreceptor is very controversial in the substantia nigra which contains high concentrations of GABA and receives important GABAergic input from the striatum and the globus pallidus. While some investigators suggested that inhibition of GABA release was not mediated by GABA<sub>A</sub> receptors (Arbilla et al., 1979; Floran et al., 1988), others thought the inhibition of GABA release was due to activation of GABA<sub>B</sub>
receptors (Giralt et al., 1989). For this reason, the role and existence of GABAβ autoreceptors in the substantia nigra remains to be determined.
12) HEMOGLOBIN:

In the human body, approximately $7.5 \times 10^{21}$ molecules of hemoglobin, an oxygen-transport protein, are present. The concentration of hemoglobin in erythrocytes of adult human is about 4.5 mM.

In LTP, hemoglobin has been widely used as a NO scavenger. However, its effect on LTP is controversial. While some investigators suggested that hemoglobin suppressed LTP (Haley et al., 1992; Musleh et al., 1993; O'Dell et al., 1991; Schuman and Madison, 1991), others reported the opposite (Izumi et al., 1992a; Pauwels and Leysen, 1992).

Slow hemolysis of erythrocytes with release of hemoglobin into the supernatant fluid has been reported to occur after 2 days of in-vitro incubation of blood (Asano et al., 1980; Barrows et al., 1955; Osaka, 1977; Miyaoka et al., 1976; Sonobe and Suzuki, 1978; Sasaki et al., 1979; Okwuasaba, 1981; Duff et al., 1987). After intracranial bleeding or hemorrhagic stroke, erythrocytes, which can remain in the intracranial cavity for days, are hemolysed in a similar fashion as in the in-vitro incubation (Barrows et al., 1955; Findlay et al., 1989). After 2 hours of subarachnoid hemorrhage, hemoglobin has been reported to be released from erythrocytes (Barrows et al., 1955). Because hemoglobin can remain in the cerebral spinal fluid for weeks (Barrows et al., 1955), neurons may be exposed to hemoglobin. In fact, hemoglobin has been suggested to induce cerebral vasospasm associated with subarachnoid hemorrhage (Weir, 1987; Osaka et al., 1980). Moreover, its iron content may be related to stroke- or head injury-induced epilepsy (Hammond et al., 1980).
In this section, the biochemistry, the oxygen-binding, and the NO-scavenging properties, of hemoglobin will be discussed.

12.1) Structure of Hemoglobin:

Normal hemoglobin is a tetramer which consists of two alpha globulin chains and two beta globulin chains that face one another across a central cavity. Each \( \alpha \) chain comprises of 141 amino acid residues, whereas each \( \beta \) chain has 146 amino acid residues. The \( \alpha \) and \( \beta \) subunits assemble into tightly bound heterologous dimers (\( \alpha-\beta \)) which in turn associate into tetramers. Each globin chain (\( \alpha \) or \( \beta \)), which resembles the structure of myoglobin, contains a hydrophobic pocket where a heme molecule or an iron [Fe (II)] protoporphyrin IX binds tightly and allows reversible binding of oxygen (Freedman, 1977; Hill, 1976). Both tertiary and quaternary structures are ideally designed for keeping the heme iron in a ferrous state which permits the loading and unloading of the oxygen molecules at the physiological partial pressures present in the blood.

The tertiary structure of each hemoglobin subunit is composed of eight \( \alpha \)-helixes labeled A to H. The E, F, and G, helixes as well as the CD non-helical segment delineate a hydrophobic crevice where heme is tightly bound. While the non-polar methyl and vinyl groups of heme extend into the hydrophobic interior of each subunit, the ionized carboxyl groups of heme are exposed to the aqueous environment. Six different ligands are required to hold the ferrous ion in place (Claude, 1992). Four of these are the nitrogen atoms of the porphyrin ring system. The fifth is the eighth residue in helix F which is a histidine. The sixth ligand is absent in deoxyhemoglobin, whereas oxygen in oxyhemoglobin functions as the sixth ligand. The non-polar side chains of the eleventh residue in helix E which is a valine and the
first residue in the CD non-helical segment which is a phenylalanine provide a sterically hindered hydrophobic pocket for the heme group (Claude, 1992). The histidine F8 and the phenylalanine CD1 are important for maintaining the iron atom in a ferrous state in the presence of oxygen and therefore reversible oxygenation (Rimington, 1959). Any change of these two residues can remove the functional properties of hemoglobin since reversible oxygenation cannot occur without steric hindrance in the hydrophobic pocket.

Besides stabilizing heme, the heme pocket also favors oxygen binding over CO binding. The affinity of CO for free heme in aqueous solution is 25,000 times that of oxygen (Claude, 1992; Rawn, 1989). If the affinity of CO to hemoglobin is as high as to free heme, oxygenation of hemoglobin would be impossible. For this reason, it is necessary to lower the affinity of CO to hemoglobin. In free heme, the bond angle between carbon monoxide and iron is nearly at right angle to the plane of free heme (Claude, 1992; Rawn, 1989). In hemoglobin, steric hindrance caused by histidine E7 and valine E11, which are located at the distal part of the heme pocket, bend and, therefore, weaken the CO-iron bond (Claude, 1992; Rawn, 1989). On the contrary, steric hindrance does not influence the iron-oxygen bond because it is naturally bent. In addition to the steric hindrance, CO does not form a hydrogen bond with the imidazole nitrogen of the histidine E7 while O2 does. As a result of these structural arrangements, hemoglobin can preferentially bind to O2.

12.2) Metabolism of Hemoglobin:

12.2.1) Synthesis of Hemoglobin:

The formation of aminolevulinic acid by the condensation of glycine and succinyl-coenzyme A in the mitochondria is a rate-determining step and the first step
in heme synthesis (Freedman, 1977; Safer, 1978). The final steps in heme synthesis, which are mediated by coproporphyrinogen oxidase and heme synthetase, also occur in the mitochondria. The heme synthesis is regulated by end-product inhibition of heme synthetase and end-producer repression of heme synthesis (Freedman, 1977, Jacob et al., 1969; Rimington, 1959). A mutual interdependence of heme and globin synthesis has been suggested (Bruns and London, 1965; Freedman, 1977; Hunt, 1976; Jacob et al., 1969; Morris and Liang, 1968; Rabinovitz, 1974; Rimington, 1959). Low concentrations of heme have been reported to reduce globin synthesis (Bruns and London, 1975; Freedman, 1977; Gross and Rabinovitz, 1972a; Morris and Liang, 1968). Moreover, heme has been shown to be necessary for globin synthesis in intact reticulocytes (Bruns and London, 1975; Freedman, 1977; Gross and Rabinovitz, 1972a; Gross and Rabinovitz, 1972b).

12.2.2) Catabolism of Heme:

Heme is catabolized by the heme oxygenase through oxidative degradation (Brown and Grundy, 1977). The heme oxygenase, which is present in the microsomal membranes of liver, kidney, brain, spleen, and bone marrow, is specific for heme, the α and β chains of hemoglobin, methemalbumin, and methemoglobin (Maines, 1977; Tenhunen et al., 1968). Heme groups of hemoglobin are eventually broken down into bilirubin in vivo (Barrows et al., 1955). Divalent cations such as cobalt, chromium, manganese, iron, copper, zinc, and lead have been shown to increase heme oxygenase activity (Maines and Kappas, 1974; Maines and Kappas, 1975; Maines, 1977).
12.3) Oxygenation of Hemoglobin:

12.3.1) Oxygen Binding and Conformational Change in Hemoglobin:

When hemoglobin changes from its deoxy state to the oxy state, one α-β dimer rotates by 15° relative to the other α-β dimer in hemoglobin (Dickerson and Geis, 1983). Oxygenation of Hemoglobin is a cooperative process. Once an O₂ molecule is bound, the binding of the succeeding O₂ molecules is facilitated so that they can bind more readily. The oxygen affinity of hemoglobin depends on the pH (German and Wyman, 1937). The Bohr effect states that hemoglobin has lower affinity for O₂ at lower pH values. The oxygen binding curve shifts to the right with decreasing pH so that O₂ can be unloaded from oxyhemoglobin more readily when muscle acidity indicates that more O₂ is required for metabolic reactions.

In the absence of oxygen, the four heme iron atoms in Hb A are in high-spin ferrous state [Fe(II)] with four unpaired electrons and one pair of electrons on each iron atom. In the presence of oxygen, these heme iron atoms are converted to a low-spin, diamagnetic ferrous state. The change from the high to the low spin state is due to unfavorable interaction of the electrons of O₂ with the unpaired electrons in the high spin state. As a result of oxygenation, the bond between histidine F8 and the ferrous ion becomes shorter and the interactions between the iron atom and the porphyrin nitrogen atoms become strengthened and more covalent in nature (Antonini and Brunori, 1971; Bunn and Forget, 1986; Dickerson and Geis, 1983; Edsall, 1972; Ho et al., 1982a, Perutz, 1989, 1990).

In addition to the change in the spin state of the iron atom, oxygenation also produces steric effects that alter the structure of hemoglobin. In deoxyhemoglobin, steric repulsion between the nitrogen atoms of the porphyrin and histidine F8 along
with the electronic repulsion between the \( \pi \) electrons of the porphyrin and the orbitals of the ferrous ion causes the iron atom to be about 0.06 nm out of the plane of the porphyrin ring (Rawn, 1989). Upon oxygenation, the change in the electronic configuration of the ferrous ion causes the iron atom to move toward the plane of the porphyrin ring by about 0.039 nm (Rawn, 1989). Not only is the iron atom moved upon oxygenation, but also tyrosine HC2 and valine FG5. The movement of these two residues disrupts the ion pairs which cross-link the chains of deoxyhemoglobin and therefore allows binding of oxygen to the heme group. When oxygen binds to the ferrous ion of one subunit, the binding of the ferrous ions of other subunits will be facilitated because the other subunits are less constrained to undergo the conformational changes associated with oxygen binding.

12.3.2) 2,3-Diphosphoglycerate (DPG) and Oxygen Affinity of Hemoglobin:

The 2,3-DPG binds to the deoxygenated hemoglobin tetramer at the central cavity between the two \( \beta \) chains in a molar ratio of one. DPG binds electrostatically to Val NA1, His NA2, and His H21 on both \( \beta \)-chains as well as Lys EF6 on one of the \( \beta \)-chains (Perutz and Imai, 1980). The valine residue at position NA1 is important for DPG binding since valine allows the complete release of the initiator methionine and prevents the acetylation of the \( \alpha \)-NH\(_2\) group by the erythrocytic N-\( \alpha \)-acetyltransferase which would suppress two of the seven DPG binding sites per tetramer (Perutz and Imai, 1980). Because DPG stabilize the conformation of deoxyhemoglobin, the oxygen affinity of hemoglobin is lowered. The conformational changes involved in oxygenation disrupt the DPG binding site such that DPG cannot bind to oxyhemoglobin. In blood, DPG facilitates oxygen unloading by shifting the oxygen binding curve to the right.
13. HEMOGLOBIN AND NITRIC OXIDE:

13.1) Nitrosylhemoglobin:

NO, which binds to the sixth coordination position of the heme in ferrohemoglobin, possesses an extremely high affinity for the heme in ferrohemoglobin. The affinity of NO to hemoglobin is approximately 3000 times that of CO (Gibson and Roughton, 1957). NO derivatives of isolated α and β subunits of human adult hemoglobin are dissimilar (Hille et al. 1977). In the presence of oxygen, nitrosylhemoglobin dissociates into methemoglobin and nitrates (Kon et al., 1977; Yoshida et al., 1980). Methemoglobin is then reduced to ferrous hemoglobin by methemoglobin reductase in erythrocytes. While majority of nitrates produced by the conversion of nitrosylhemoglobin to oxyhemoglobin are excreted in urine, some of these nitrates can also be discharged into the oral cavity, where they are transformed to nitrites, through the salivary glands (Yoshida and Kasama, 1987). In general, most of the metabolites of inhaled NO are excreted rapidly from the body within 48 hours (Yoshida et al., 1978). Because the regeneration of oxyhemoglobin is much faster than the dissociation of NO from nitrosylhemoglobin, this regenerative process plays a protective role in NO intoxication.

13.2) Nitric Oxide and Long Term Potentiation (LTP):

NO synthase has been reported to be present in hippocampal interneurons located in the stratum oriens, the pyramidal cell layer, and the stratum radiatum (Leigh et al., 1990; Mizukawa et al., 1989; Mufson et al., 1990; Seidel et al., 1991; Vincent and Hope, 1992; Vincent and Kimura, 1992). Majority of neurons in the medial septum and the nucleus of the diagonal band of Broca, which project to the
hippocampus (Kinjo et al., 1989), also possess NO synthase (Kinjo et al., 1989; Mizukawa et al., 1989; Pasqualotto and Vincent, 1991; Schöber et al., 1989).

NO has been suggested to be a possible retrograde messenger mediating LTP (Garthwaite et al., 1988). In addition, NO has also been thought to mediate NMDA action in the hippocampal formation (Gally et al., 1990) because L-NAME, a nitric oxide synthase inhibitor, prevented the L-arginine-induced electroencephalogram desynchronization and potentiation of the epileptogenic effects of NMDA (Mollace et al., 1991). In vivo, the increase in cGMP levels induced by local injections of NMDA into the hippocampus were blocked by a NO synthase inhibitor (Wood et al., 1992). In hippocampal slices, cGMP production induced by the activation of the NMDA receptor was also suppressed by NO synthase inhibitors (East and Garthwaite, 1991; Foster and Roberts, 1981). These observations support the speculation that NO is involved in mediating NMDA action.

Studies on the role of NO in the induction of LTP have been conducted by many groups (Böhme et al., 1991; Bon et al., 1992; Haley et al., 1992; Izumi et al., 1992b; Musleh et al., 1993; O'Dell et al., 1991; Schuman and Madison, 1991). NO synthase inhibitors and hemoglobin which is known to be a membrane impermeant NO scavenger (Gibson and Roughton, 1957) have been commonly used in these studies. The role of NO in the induction of LTP is controversial. While some investigators have reported that LTP could be suppressed by NO synthase inhibitors and hemoglobin (Böhme et al., 1991; Bon et al., 1992; Haley et al., 1992; Musleh et al., 1993; O'Dell et al., 1991; Schuman and Madison, 1991), others conclude differently (Izumi et al., 1992b; Kato and Zorumski, 1993; Williams et al., 1993).
This discrepancy can be attributed to the assumption that the inhibition of the induction of LTP by oxyhemoglobin or NO synthase inhibitors means an involvement of NO or NO synthase, respectively. Oxyhemoglobin may have effects independent of NO inhibition such as the inhibition of acetylcholinesterase (Linnik and Lee, 1986). On the other hand, NO synthase inhibitors, which are arginine analogs, may inhibit protein and peptide synthesis, postranslational arginylation (Hallak et al., 1991), arginase, arginosuccinase, \( \text{Na}^+/\text{K}^+ \)-ATPase (Nagai et al., 1985), the formation of thrombin, guanidinoacetate, and creatine, or arginine transport into cells (Bogle et al., 1992). Furthermore, the direct electrophysiological actions of NO synthase inhibitors and hemoglobin on neurons have not been examined. It is possible that mechanisms other than chelation of NO and suppression of NO synthesis are involved in the suppression of LTP. For these reason, the role of NO in LTP remains to be resolved.
14) HEMOGLOBIN AND CEREBRAL VASOSPASMS:

Vasospasm has been reported to start and subside 3 days and 14 days after subarachnoid hemorrhage (SAH), respectively (Weir et al., 1978). Maximal vasospasm usually occurs 6 to 7 days after the hemorrhage (Weir et al., 1978). While intense polymorphonuclear cell infiltration of the meninges takes place within 24 hours after SAH, breakdown of RBCs occurs by 16 to 32 hours after the hemorrhage (Bagley, 1928; Hammes, 1944; Alpers and Forster, 1945). Even though hemolysis of RBCs peaks around day 7, intact RBCs can be present in the arachnoid space up to 35 days after SAH (Bagley, 1928; Hammes, 1944; Alpers and Forster, 1945). Using absorption spectrophotometry to examine cerebrospinal fluid after SAH, oxyhemoglobin has been reported to appear 2 hours after SAH (Barrows et al., 1955). Although the amount of oxyhemoglobin continuously decreased while that of bilirubin increased, the disappearance of both pigments did not occur until 2 to 3 weeks after SAH (Barrows et al., 1955). These observations show that oxyhemoglobin is present during vasospasm and it may be the mediator of SAH-induced vasospasm (Simmonds, 1953; Dupont et al., 1961).

Even though substances such as serotonin, biogenic amines, peptides, and eicosanoids may attribute to SAH-induced vasospasm, antagonists of these vasoconstrictors including atropine, methysergide, cinanserin, ketanserin, phenoxybenzamine, phentolamine, mepyramine, chlorpheniramine, propranolol, salbutamol, angiotensin, sarcosine, alanine, theophylline, and quinine, are unable to reverse oxyhemoglobin-induced contractions of cerebral arteries in vitro and in vivo (Cook et al., 1979; Fujiwara et al., 1986; Fujiwara and Kuriyama, 1984; Iwai et al., 1988; Kanamaru et al., 1987; Kajikawa et al., 1979; Nakagomi et al., 1988; Nakayama
et al., 1989; Ohmoto et al., 1979, Ohta et al., 1980a, b; Okamoto, 1982, 1984; Onoue et al., 1989; Ozaki and Mullan, 1979; Tanishima, 1980; Toda, 1980). However, agents such as papaverine, calcium channel antagonists, and some inhibitors of eicosanoids synthesis have been reported to relax oxyhemoglobin-induced vasospasm (Cook et al., 1979; Fujiwara et al., 1986; Fujiwara and Kuriyama, 1984; Kanamaru et al., 1987; Ohta et al., 1980b; Onoue et al., 1989; Tanishima, 1980). Oxyhemoglobin, which has been observed to increase the intracellular concentration of inositol phosphates that are second messengers involved in smooth muscle contraction (Vollrath et al., 1990), can act via a variety of pathways over a prolonged period to produce arterial narrowing and ultrastructural damage to arteries after SAH. Vasospasm induced by oxyhemoglobin may involve direct effects on smooth muscle, release of vasoactive eicosanoids and endothelin (Machi et al., 1991; Masaoka et al., 1990; Sato et al., 1990; Shigeno et al., 1990) from the arterial wall, inhibition of endothelium-dependent relaxation (Toda et al., 1988; Tsuji et al., 1975), production of bilirubin (Duff et al., 1988; Miao and Lee, 1989) and lipid peroxides (Asano et al., 1980; Sasaki et al., 1979), and damage to perivascular nerves (Okada et al., 1980; Linnik and Lee, 1989; Lee et al., 1984). However, the relative contributions of these mechanisms of action of oxyhemoglobin to SAH-induced vasospasm are yet to be determined.
15) **Hemoglobin and Epilepsy:**

15.1) **Nomenclature of Epileptic Seizures:**

Epileptic seizures can be categorized into generalized and partial seizures. While generalized seizures often involve both cerebral hemisphere and alter consciousness, partial seizures only affect a part of one cerebral hemisphere. Partial seizures can be further classified into simple and complex types. Simple partial seizures consist of focal neurologic events with intact consciousness, whereas complex partial seizures involve focal events with impaired consciousness. Because seizure activity in the cerebral cortex can spread, simple partial seizures may develop into complex partial seizures. Both simple and complex partial seizures may also evolve into secondarily generalized seizures.

In addition to the above classification, epileptic seizures are divided into primary and secondary types. While primary or idiopathic epileptic seizures are usually inherited, age-related, benign, and unassociated with identified structural lesions, secondary (symptomatic) epileptic seizures are caused by an identifiable underlying disease or lesion.

15.2) **Pathogenesis of Epileptic Seizures:**

Epilepsy is characterized by recurrent epileptic seizures which result from paroxysmal and abnormally synchronous discharges of cerebral cortical neurons caused by an imbalance of excitatory and inhibitory synaptic processes (Engel, 1990). The imbalance between the two synaptic processes can occur as a result of artificial electrical stimulation of brain tissue, metabolic disorders, hypoglycemia, and traumatic brain injury. In secondary epileptic seizures, loss of neurons, synaptic reorganization, and the balance between levels of glutamate and GABA are the common causes.
15.3) Subarachnoid Hemorrhage-Induced Epileptic Seizures:

Posttraumatic epilepsy is often observed in patients with cerebral hematoma, cortical laceration, or subarachnoid hemorrhage (Caveness, 1963; Caveness and Liss, 1961; Jennett, 1975; Kaplan, 1961; Richardson and Dodge, 1954; Russell and Whitty, 1952, 1953; Ward, 1972). Under normal physiological conditions, biological iron, which is normally bound in transferrin and hemoglobin, is released into brain tissues by vascular endothelium (Harrison, 1971; Kristensson and Bornstein, 1974; Rigby, 1971; Strassmann, 1945). In head injury or hemorrhagic cortical infarction, extravasation of blood and deposition of iron within the neutrophil can occur (Kaplan, 1961). Because topical cortical application of hemolyzed red blood cells (Levitt et al., 1971) or intracortical injection of ferrous or ferric chloride (Willmore et al., 1978a, b, c) have been reported to generate seizures, blood or its metabolites may play a role in generation of epileptic seizures induced by intracranial hemorrhage. Indeed, focal epileptiform paroxysmal discharges were reported after intracortical injection of whole blood, hemolyzed erythrocytes, methemoglobin, ferritin, ferrous chloride, ferric chloride, fibrinogen, hemin, and cottonoid in cats and guinea pigs (Hammond et al., 1980). Besides epileptic seizures, iron deposition, loss of neurons, and glial proliferation have also been observed at the site of brain lesions (Hammond et al., 1980; Rand and Courville, 1945; Pollen and Trachtenberg, 1970). Because hemoglobin and iron liberated from hemoglobin have been implicated in generating oxygen free radicals, peroxidation of neuronal membranes may occur as a result of the generation of oxygen free radicals (Mori et al., 1990).
15.4.) *Iron-Induced Epilepsy:*

Injection or ionophoretic deposition of iron salts into rodent has been shown to cause acute epileptiform discharges, formation of focal cerebral edema, cavitory necrosis, and the occurrence of chronic or recurrent seizures (Willmore et al., 1978a, b, c). Lipid peroxidation of neuronal membranes which may produce epileptic foci has been attributed to the generation of active oxygen-free radicals (O$_2^*$, •OH, •OOH) induced by iron (Hiramatsu et al., 1983, 1984). Besides the above phenomenon, the levels of aspartate and GABA have been reported to decrease while the levels of alanine and glycine increase in iron-induced epilepsy (Shiota et al., 1989). The decrease in aspartate and GABA levels has been attributed to the accelerated release in the acute epileptic focus (Shiota et al., 1989). Alterations of excitatory and inhibitory amino acid levels may relate to the decrease in the activity of cerebral Na$^+$/K$^+$-ATPase which can cause brain edema accompanied by convulsion (Shiota et al., 1989).

Using electrocorticographic characteristics, three stages have been identified in seizures induced by injection of iron into rat cerebral cortex (Moriwaki et al., 1992). The first stage includes an increase in the frequency of isolated spikes, which are often present on one side of the cortex, in the first 30 days after the injection. The second stage occurs when an increase in the number of spike and wave complexes, which exhibit a bilateral appearance, is noted between 30 days and 6 months after the injection. In the third stage, the proportion of the brain showing spike and wave complexes remains almost constant. In addition, alterations in the cerebral cortex such as changes in the adenosine- or norepinephrine-sensitive cAMP generating systems of the cortices probably occur in this stage. Similar to Moriwaki et al.,
Mizukawa et al. (1991) also observed 3 stages of responses induced by iron injections by using c-fos-immunohistochemistry. At 3 hours after the iron administration, c-fos-immunopositive neurons were observed in the dentate gyrus, CA1, CA2, the vicinity of the iron injected cerebral cortex, and amygdala. While several heavily stained cells and nerve fibers appeared around the injection site three days after the administration, large cavities and gliosis were present at three weeks after the injection.
16) MATERIALS AND METHODS:

16.1) Animal Source:

Male Wistar Rats (100-150g) provided by the Animal Care Center of the University of British Columbia were used in all studies. After the animals reached the Department of Pharmacology & Therapeutics of the University of British Columbia, they were kept in a wire cage (dimensions: 17" X 7" X 9.75") in the animal room. Animals came in a shipment of six animals. Each shipment of animals was stored in separate cages. The temperature of the animal room was carefully controlled and kept between 20 to 22°C. Lights of the room were kept on from 6 a.m. to 6 p.m. daily. Chow contained in a compartment attached to the wire cage and water contained in an inverted 250 ml bottle were available to the animals. Food and water supplies were refilled daily from Monday to Friday. On every Friday, extra food and water were supplemented to maintain the animals' diet through the weekend. The excretion trays of the wire cages were cleaned and replaced once every two days.

16.2) Slice Preparation:

Rats were transferred from the animal room to the laboratory in a plastic cage (dimensions: 11.5" X 5" X 7.25"). The animals were then placed on ice in a glass chamber in order to lower their metabolic rate. Inside the chamber, animals were anesthetized with 2% halothane and carbogen (95% O₂, 5% CO₂) until surgical anesthesia was achieved (i.e. the eyeblinking and the withdrawal reflexes are absent, and the respiration is thoracic in origin.).

After surgical anesthesia was acquired, a medial incision was made at the top of the animal's head in order to expose the skull. A pair of nippers was used to create a small incision at the base of the animal's skull. Through this incision, a pair of scissors
was manipulated to cut along the sagital suture line. A pair of ronguers was subsequently used to remove the bone and the dura. The brain was gently removed from the intracranial cavity by a spatula. Once the brain was removed from the intracranial cavity, it was immediately washed with cold (4°C) ACSF which was saturated with carbogen. The hippocampi were exposed by removing the two cerebral hemispheres laterally. One of the hippocampi was peeled away from the remaining portion of the brain. The hippocampus was then transferred to the cutting platform of a McIlwain tissue chopper and positioned such that the septo-temporal axis of the hippocampus was perpendicular to the blade of the chopper. The hippocampus was cut transversely into slices of 400 μm in thickness. The slices were then separated by a pair of spatulas in a petri dish containing cold oxygenated ACSF and stored in a holding chamber which was continuously oxygenated by carbogen.

The CA3 region of all slices was removed. Slices were allowed to equilibrate with the superfusate for at least one hour before any electrophysiological recordings were made. Slices stored in this manner were viable for eight hours post-surgery. At the end of the equilibrium period, only slices with a discrete cell line were used for electrophysiological recordings. In order to avoid the slice from moving in the recording chamber, the slice was placed in between two nylon meshes which are mounted on two separate overlapping plastic rings. The meshes were manipulated such that the slice was secure between the nets without any physical damages.

16.3) **Slice Chamber:**

The slice chamber comprised of a rectangular plexi glass block, the superfusing chamber, the inlet, the suction, and the oxygen apertures (Fig. 2). The superfusing chamber resembles the shape of a key hole. While the circular section of the superfusing...
chamber is located close to the inlet aperture, the narrow or rectangular section is closer to the suction aperture. The main inflow line was fed into the inlet aperture, whereas a suction tube for maintaining a constant circulation of superfusate was fed through the suction aperture and into the superfusing chamber. Before the main inflow line reached the inlet aperture, it coursed through an aluminum heating block which was located underneath the slice chamber. The temperature of the superfusate was carefully controlled and maintained at a constant temperature by a feed-back temperature sensing device in the control panel of the heater.

A ground wire, which has a silver pellet at its end, was secured on the plexi glass block by plasticine. The silver pellet of the ground wire was placed inside the rectangular section of the superfusing chamber.

The main inflow line is divided into seven different inlets by a manifold. Each inlet was connected to a 60 ml barrel. The barrels were adjusted to a height where a flow rate of about 1.6 ml/min was obtained.
Figure 2. Slice chamber for electrophysiological recordings.
16.4) **Superfusing Media:**

Slices were superfused with oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid containing (in mM) 120 NaCl, 3.1 KCl, 26 NaHCO₃, 2 MgCl₂, 2 CaCl₂, 10 dextrose (pH 7.4) at a flow rate of about 1.6 ml/min. In some experiments, CNQX (Tocris Neuramin), APV (Sigma), TTX (Sigma), picrotoxinin (Sigma), rat or bovine hemoglobin (Sigma), ferric chloride (FeCl₃; Nichols), tri-sodium-citrate (B.D.H.), or N⁰-nitro-L-arginine (Sigma) was added to the superfusing medium. Concentrated stock solutions of CNQX, APV, and TTX were prepared in distilled water and diluted with ACSF to the desired concentration. Rat or bovine hemoglobin, ferric chloride, tri-sodium-citrate, and N⁰-nitro-L-arginine, which were in powder form, were directly dissolved in the superfusing medium to acquire the desired concentration. In cases where the total concentration of divalent cations was significantly altered, an appropriate amount of calcium ions were removed from the recipe of the superfusing medium to compensate the change in the total concentration of divalent cations. The pH of all oxygenated superfusates was about 7.4.

In some experiments, a Ca²⁺-free medium was prepared by omitting calcium chloride from the recipe. Even though contamination from other chemicals was possible, the amount of Ca²⁺ contributed by other chemicals was likely to be insignificant.

All superfusing media were oxygenated by carbogen before and during applications. The main barrel which contained the principal superfusing medium was continuously supplied with the medium from a reservoir located above the barrel. In order to minimize dead space in the superfusing tubings, air bubbles trapped in the lines were removed, before each experiment, by a suction device. All filled lines which were not in use were temporarily closed by butterfly clips. Whenever a particular filled line was needed, the butterfly clip of that line was removed while another butterfly clip was
simultaneously placed on the previously running line. By doing this, the slice chamber received rapid exchange of different media with minimal flow disturbance.

16.5) Recording and Stimulating Equipment:

The superfusing chamber, as well as the stimulating and recording electrodes were all mounted on an aluminum plate. The base plate was in turn placed on a vibration-free table. An aluminum wire-cage was used to shield the set up from electrical noises. All recording and stimulating equipment were mounted on two racks which are located to the right of the vibration-free table. The superfusing barrels were mounted on a stand which was positioned to the left of the table. A Zeiss gross dissecting microscope mounted on a movable stand was also situated on the same side as the superfusing barrels.

16.5.1) Stimulators and Isolation Units:

A Grass S88 stimulator which were connected to two Grass PSIU6 photoelectric stimulus isolation units with constant current output was used in all experiments. While one unit fed square wave current pulses or DC current through the Axoclamp into the recording microelectrode, the other unit supplied current to the stimulating electrode.

16.5.2) Amplifiers:

Both extracellular and intracellular potentials were recorded with an Axoclamp 2A microelectrode clamp (Axon Instruments) in the current clamp (CC) mode. A precision resistor of 100 MΩ in resistance, which were located in the headstage, set the headstage current gain (H) to be 0.1. At this value, the range of the bridge balance was 0 to 1000 MΩ; the maximum DC current command was ± 10 nA; and the range of the capacitance neutralization was -1 to 4 pF. The signals from Axoclamp were amplified ten times and filtered between 0.1 kHz and 1 kHz before they reached the recording systems.
The null-bridge method was used to determine the resistance of the microelectrodes. When the microelectrode was placed in the superfusing medium, the voltage response elicited by a 0.2 nA and 200 ms square pulse stimulation was removed by adjusting the bridge balance of the Axoclamp. The value registered on the bridge balance was the resistance of the microelectrode. After the microelectrode impaled a neuron, the voltage deflection caused by the same square pulse was once again eliminated by further adjustment on the bridge balance. The value of the bridge balance after the second adjustment was the sum of the resistance of the microelectrode and that of the cell membrane. At the end of each experiment, the above processes were performed in reverse order to ensure that the resistance of the recording microelectrode was not altered in the experiment.

16.5.3) Recording Systems:

Intracellularly recorded potentials were monitored by a Tetronix type 564 storage oscilloscope and analyzed by a DATA 6000 waveform analyzer. Each recording from the DATA 6000 waveform analyzer represented an average of four consecutive records. While the recordings from the Data 6000 waveform analyzer were recorded by a HP7470-A graphic plotter (Hewlett Packard), the responses from the oscilloscope were recorded by a Grass 79D polygraph.

Extracellularly recorded potentials were recorded in a similar manner. However, each recording from the DATA 6000 waveform analyzer only represented an average of two consecutive responses.
16.5.4) Electrodes:

16.5.4.1) Recording Electrodes:

Both Intracellular and extracellular recording electrodes were made from standard wall borosilicate glass capillaries supplied by Sutter Instrument. The microelectrodes were pulled by a programmable Flaming/Brown P-87 model micropipette puller (Sutter Instrument). A 2 mm wide horizontal trough filament (Sutter Instrument) was used.

The inner and outer wall diameters of the extracellular electrodes were 1.17 and 1.5 mm, respectively. The program setting used to pull the extracellular electrodes was: Heat = 390 units; Pull = 0 units; Velocity = 86 units; Time = 255 units. The electrodes were filled with ACSF. The tip resistance of these electrodes was about 5 MΩ.

The inner and outer wall diameters of the intracellular electrodes were 1.0 and 0.58 mm, respectively. These electrodes were pulled with the following setting: Heat = 390 units; Pull = 250 units; Velocity = 80 units; Time = 160 units. A 3M potassium acetate electrolyte was used in most intracellular recordings. The tip resistance of the intracellular microelectrodes ranged from 90 to 110 MΩ.

16.5.4.2) Stimulating Electrodes:

A bipolar concentric stimulating electrode (SNEX-100; DKI) was used in all experiments. The shaft length and the contact length of the electrode was 50 mm and 0.75 mm, respectively. The contact diameter of the electrode was 0.1 mm. The resistance of the electrode was around 1 MΩ.

16.5.4.3) Positioning of Electrodes:

In extracellular studies, the recording electrode was manipulated in the X, Y, and Z planes by an Optikon micropositioner. In intracellular studies, a DKI 650 hydraulic
micropositioner, which could lower the electrode by 2.5 μm in 2.5 x 10^-3 ms, was used in addition to the Optikon micropositioner. The stimulating electrode was installed on a DKI electrode carrier which also allowed the stimulating electrode to be moved in a three-dimensional manner.

**16.6) Electrophysiological Recordings:**

**16.6.1) Extracellular Recordings:**

Field EPSPs evoked by stimulation (0.05 Hz; 0.2 ms; 10-150 μA) of the stratum radiatum with a bipolar electrode (SNEX 100; Rhodes Electronics) were recorded next to the CA1 cell body region. The stimulation strength was adjusted to produce field EPSPs of 0.5 to 1 mV in size. Fifteen minutes of stable responses were obtained as control values before any drug applications.

**16.6.2) Intracellular Recordings:**

Intracellular responses evoked by stimulation (0.5 Hz; 0.2 ms; 10-150 μA) of the stratum radiatum with a bipolar electrode (SNEX 100, Rhodes Electronics) were recorded in CA1 neurons of the hippocampus. The input resistance of neurons was examined by measuring the change in membrane potential caused by rectangular hyperpolarizing current pulses (-0.05 to -0.2 nA, 200 ms) injected through the recording electrode. Excitability of the neurons was monitored by examining the number of spikes evoked by rectangular depolarizing current pulses (0.05 to 0.3 nA, 200 ms). The stimulation strength was adjusted to produce EPSPs of 5 to 10 mV in size and IPSPs of 2 to 5 mV in amplitude. Fifteen minutes of stable EPSPs and IPSPs were collected as control responses before any drug applications. The membrane potential, the input resistance, and the depolarization-induced discharge of action potentials were also ensured to be stable during this control period.
In some experiments, neurons were current-clamped to compensate for the drug-induced change in membrane potential (i.e. bringing the membrane potential back to control level) when records of the EPSPs, the IPSPs, the input resistance, and the depolarization-induced discharge of action potentials were taken during drug application. These records were then compared to the control responses at the same membrane potential.

16.7) Analysis of Extracellular and Intracellular Recordings:

16.7.1) Extracellular Recordings:

The height of the extracellularly recorded EPSPs was determined by the distance measured from the peak of the response to the baseline. The distance was then multiplied by a proper conversion factor to express the actual size of the response in millivolts. The initial slope of the extracellularly recorded EPSPs was also computed and expressed in mV/ms by the DATA 6000 waveform analyzer.

16.7.2) Intracellular Recordings:

The amplitude of the EPSPs and the IPSPs was measured by the distance between the baseline and the peak of the EPSPs, and those between the peaks of the IPSPs and the baseline, respectively. The actual size of the EPSPs and the IPSPs in millivolts were obtained by multiplying the distances by a conversion factor. The latencies of the control synaptic responses (i.e. the durations measured from the stimulation artifact to the peak of the EPSP, the fast IPSP, and the slow IPSP) were used as references for subsequent recordings. The initial slope of the EPSP was also computed and expressed in mV/ms by the DATA 6000 waveform analyzer.
16.8) **Statistics:**

All control and post-drug synaptic responses were normalized to the mean of the control responses. All data were expressed as mean ± S.E.M.. Two tailed paired sample t-test was used to determine the statistical significance of the effect of the drug on the input resistance, the change in membrane potential, and the depolarization-induced discharge of action potentials. The statistical significance of the drug on the synaptic responses at any time was determined by ANOVA and the Duncan test, a post-hoc multiple comparison analysis. In both statistical tests, the level of significance (ρ) was arbitrarily selected to be 0.05. In other words, a probability of less than 0.05 was considered to be statistically significant.
17) EXPERIMENTAL PROTOCOL:

17.1) Electrophysiological Effects of Rat Hemoglobin on Rat Hippocampal CA1 Neurons:

17.1.1) Without Compensation of the Hemoglobin-Induced Change in Membrane Potential:

Rat hemoglobin (0.05 mM) was prepared by dissolving the appropriate amount of hemoglobin powder (Sigma) in ACSF. After the EPSP, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were observed to be stable for 15 minutes, oxygenated rat hemoglobin (0.05 mM) was applied for 15 minutes. After the hemoglobin application, the slice was reperfused with ACSF for 30 minutes. During drug application and reperfusion, the EPSP, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were measured quantitatively.

17.1.2) With Compensation of the Hemoglobin-Induced Change in Membrane Potential:

During depolarization, while the input resistance of neurons may increase as a result of closures of voltage-dependent ion channels, the EPSP may be suppressed as the reversal potential of the EPSP is approached. Since the EPSP and the input resistance could be changed by the hemoglobin-induced change in membrane potential, the membrane potential was current clamped to the pre-hemoglobin level when the EPSP and the input resistance were measured during drug application. In cases where the membrane potential during reperfusion was not the same as that of the control period, compensation of the change in membrane potential was also done to allow comparison of the responses measured during reperfusion to the control responses.
17.2) **Electrophysiological Actions of Bovine Hemoglobin on Rat Hippocampal CA1 Neurons:**

The effects of rat hemoglobin (0.05 mM; Sigma) were compared to those of bovine hemoglobin (0.05 mM; Sigma). There was no significant difference between the actions of rat and bovine hemoglobins on the membrane potential, the input resistance, and the EPSP. Because bovine hemoglobin is much more economical, and there are no known differences between rat and bovine hemoglobins, bovine hemoglobin was used in subsequent experiments.

17.2.1) **Without Compensation of the Hemoglobin-Induced Change in Membrane Potential:**

Different concentrations of bovine hemoglobin (0.01 to 1 mM) were used to determine the appropriate concentration to induce significant effects consistently in CA1 neurons. Bovine hemoglobin was prepared by dissolving the appropriate amount of hemoglobin powder (Sigma) in ACSF to reach the desired concentration. After the synaptic transients, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were observed for 15 minutes for stability, oxygenated hemoglobin was applied for 15 minutes. Following the hemoglobin application, the slice was reperfused by ACSF for 30 min. During drug application and reperfusion, the synaptic transients, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were measured quantitatively.
17.2.2) With Compensation of the Hemoglobin-Induced Change in Membrane Potential:

If the membrane potential was changed during hemoglobin application or reperfusion, the membrane potential was current clamped to the control value when the electrophysiological responses were measured. In some experiments, 50 µM of picrotoxinin was included in the superfusing medium in order to examine the effects of hemoglobin on GABAergic-mediated slow IPSPs without contamination by the fast IPSPs.

17.3) The Current-Voltage (I/V) Relationship for Bovine Hemoglobins:

Like the synaptic transients, the input resistance can also be changed by a change in membrane potential. In order to assure that the hemoglobin-induced change in input resistance was not due to the change in membrane potential caused by hemoglobin, the I/V relationship for hemoglobin was studied with and without compensation to the hemoglobin-induced change in membrane potential in the same neuron.

17.3.1) Without Compensation of the Hemoglobin-Induced Change in Membrane Potential:

Voltage deflections elicited by depolarizing and hyperpolarizing rectangular current pulses (0.1 nA to -0.2 nA) injected through the recording electrode were measured during control and during hemoglobin application. For hyperpolarizing pulses, the membrane potentials at the peaks of the current pulse-induced voltage responses were calculated by subtracting the absolute values of voltage deflections elicited by the current pulses from the membrane potential recorded when the current pulses were injected. For depolarizing pulses, the membrane potentials at the peaks of the current pulse-induced voltage responses were calculated by adding the absolute values of the voltage deflections elicited by the current pulses to the membrane potential recorded when the current pulses were injected. The membrane potentials at the peaks of the current pulse-induced
voltage deflections and the corresponding current strengths measured during control and hemoglobin application (0.1 mM; 15 min) were then plotted on a voltage vs current curve. The input resistance of the neuron in control medium and in hemoglobin was represented by the slope of the respective graph.

17.3.2) With Compensation of the Hemoglobin-Induced Change in Membrane Potential:

During hemoglobin application (0.1 mM; 15 min.) and reperfusion, the membrane potential was current clamped to the control value when the depolarizing and hyperpolarizing current pulses were injected through the recording electrode. While the membrane potentials at the peaks of the depolarizing current pulse-induced voltage deflections were calculated by adding the absolute values of the current pulse-induced voltage deflections to the control membrane potential, those at the peaks of hyperpolarizing current-induced voltage deflections were computed by subtracting the absolute values of the current pulse-induced voltage deflections from the control membrane potential. The absolute membrane potentials and the respective current strengths were then plotted on a voltage vs current graph.

17.4) Calcium and the Hemoglobin-induced Depolarization:

In order to determine if the depolarizing action of hemoglobin was Ca\(^{2+}\)-dependent, a Ca\(^{2+}\)-free medium containing 10\(^{-7}\) M of TTX, 3.5 mM Mg\(^{2+}\), and 0.5 mM Mn\(^{2+}\) was used for superfusion. The total concentration of divalent cations in the Ca\(^{2+}\)-free medium was maintained at 4 mM by increasing the concentration of Mg\(^{2+}\) from 2 to 3.5 mM and by including 0.5 mM of Mn\(^{2+}\) in the medium.
In normal ACSF containing $10^{-7}$ M TTX, a depolarization induced by superfusion with 0.1 mM of hemoglobin for 15 minutes was obtained as a control response. At the end of the application of hemoglobin, the slice was reperfused with the normal medium for 30 minutes. At the end of the reperfusion period, the normal superfusate was replaced by the Ca$^{2+}$-free medium. After superfusing the slice with the Ca$^{2+}$-free medium for 15 minutes, 0.1 mM hemoglobin prepared with the Ca$^{2+}$-free medium was applied for 15 minutes. A reperfusion with the normal medium (30 min) followed the second hemoglobin application. At the end of the second reperfusion, 0.1 mM hemoglobin prepared in the normal medium was applied for 15 minutes. After the third application of hemoglobin, the slice was reperfused with the normal medium for 30 minutes. All three hemoglobin applications were done at the same membrane potential so that the responses recorded in the normal medium (i.e. the control response and the response from the third application of hemoglobin) could be compared with that recorded in the Ca$^{2+}$-free medium at the same membrane potential.

17.5) Involvements of NMDA and Non-NMDA Receptors in the Hemoglobin-Induced Depolarization:

Besides calcium, the depolarizing action of hemoglobin may be due to the activation of glutamate receptors. In order to determine whether activation of excitatory amino-acid receptors was necessary for the depolarizing action of hemoglobin, hemoglobin was applied in the presence of APV or CNQX.
17.5.1) APV and the Hemoglobin-Induced Depolarization:

The stock solution of APV was diluted with a Mg²⁺-free medium containing \(10^{-7}\) M TTX to reach a final concentration of 50 \(\mu\)M. The concentration of the divalent cations in the Mg²⁺-free medium was maintained at 4 mM by increasing the Ca²⁺ concentration from 2 to 4 mM. A control hemoglobin-induced depolarizing response was obtained by superfusing the slice for 15 minutes with 0.1 mM hemoglobin, which was prepared with ACSF containing \(10^{-7}\)M TTX. At the end of the hemoglobin application, the slice was reperfused with the normal medium for 30 minutes. Following the reperfusion period, the normal medium was replaced with the Mg²⁺-free medium. After superfusing the slice with the Mg²⁺-free medium for 5 minutes, 0.1 mM hemoglobin prepared in the Mg²⁺-free medium was applied for 15 minutes. A 30 minutes long reperfusion with the normal medium followed the second application of hemoglobin. At the end of the second reperfusion, 0.1 mM hemoglobin prepared with the normal medium was applied for 15 minutes again. After the third application of hemoglobin, the slice was also reperfused with the normal medium for 30 minutes. All hemoglobin-induced depolarizing responses were elicited at the same membrane potential to allow comparison between the responses recorded in the normal medium (i.e. the control response and the response from the third application) and that obtained in the Mg²⁺-medium.

17.5.2) CNQX and the Hemoglobin-Induced Depolarization:

The stock solution of CNQX was diluted with a normal medium containing \(10^{-7}\) M TTX to reach a final concentration of 20 \(\mu\)M. A control hemoglobin-induced depolarizing response was obtained by superfusing the slice with 0.1 mM hemoglobin, which was
prepared with ACSF containing $10^{-7}$ M TTX, for 15 minutes. At the end of the hemoglobin application, the slice was reperfused with the normal medium for 30 minutes. Following reperfusion, the normal medium was replaced with the CNQX containing medium. After superfusing the slice with the CNQX containing medium for 10 minutes, 0.1 mM hemoglobin prepared with the same medium was applied for 15 minutes. A 30 minutes long reperfusion with the normal medium followed the second application of hemoglobin. At the end of the second reperfusion, 0.1 mM hemoglobin prepared with the normal medium was again applied for 15 minutes. After the third application of hemoglobin, the slice was also reperfused with the normal medium for 30 minutes. All depolarizing responses were elicited at the same membrane potential so that the responses recorded in the normal medium (i.e. the control response and the response from the third application) could be compared to that acquired in the CNQX containing medium.

17.6) **Glutamate-Induced and Hemoglobin-Induced Depolarizations:**

A stock solution of concentrated glutamate was diluted with ACSF containing $10^{-7}$ M TTX to reach a final concentration of 5 mM. Glutamate (5 mM) was applied for 1 minute to elicit a depolarization. Two glutamate-induced depolarizations which were similar in size were used as control responses. During the 15 minutes application of 0.1 mM hemoglobin, 5 mM glutamate was applied for 1 minute at the peak of the hemoglobin-induced depolarization. Glutamate was also applied for 1 minute at 5 and 30 minutes post-hemoglobin.

When glutamate was applied during hemoglobin application, the membrane potential was current-clamped to the control value. In general, all glutamate-induced
responses were recorded at the same membrane potential so that responses recorded before, during, and after hemoglobin-application could be compared.

17.7) Nitric Oxide Synthase Inhibitor and the Actions of Hemoglobin:

In order to determine if the actions of hemoglobin were caused by the NO scavenging property of hemoglobin, the actions of hemoglobin were examined in the presence of a nitric oxide synthase inhibitor, Nω-nitro-L-arginine. A low (100 μM) and a high (500 μM) doses of Nω-nitro-L-arginine were used in this investigation.

Nω-nitro-L-arginine was prepared by dissolving the appropriate amount of the agent powder in 20 ml of ACSF whose pH was lowered to about 1.5 by HCl. The aliquot was then diluted to the desired concentration (100 μM or 500 μM) with ACSF. The pH of the final solution with oxygenation was about 7.4. Hemoglobin (0.1 mM) was prepared with ACSF containing either 100 or 500 μM of Nω-nitro-L-arginine.

In the presence of either 100 or 500 μM of Nω-nitro-L-arginine, the synaptic transients, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were determined to be stable for 15 minutes before hemoglobin was applied. At the end of the hemoglobin application, the slice was reperfused with ACSF containing either the low or high dose of Nω-nitro-L-arginine. The membrane potential was current-clamped to the control value when the synaptic transients, the input resistance, and the depolarization-induced discharge of action potentials were recorded.
17.8) Iron and the Actions of Hemoglobin:

As a component of hemoglobin, iron could contribute to the effects of hemoglobin. In this study, the effects of iron were compared to those of hemoglobin.

A low (0.4 mM) and a high (2 mM) dose of iron which corresponded to the iron content in 0.1 and 0.5 mM of hemoglobin, respectively, were used in this investigation. The 0.4 mM iron solution was prepared by dissolving the appropriate amount of ferric chloride powder with a phosphate-free medium containing 0.4 mM of tri-sodium citrate. The concentration of NaCl was reduced from 120 to 119.4 mM to compensate for the inclusion of 0.4 mM ferric chloride and 0.4 mM tri-sodium citrate, as well as the exclusion of 1.8 mM sodium dihydrogen phosphate in the iron containing medium. Like the iron solution, the control superfusate also contained 0.4 mM tri-sodium citrate.

The 2 mM iron solution was prepared as described for the 0.4 mM counterpart. The concentration of NaCl was reduced from 120 to 109.8 mM to compensate for the inclusion of 2 mM ferric ions and 2 mM tri-sodium citrate, as well as the exclusion of 1.8 mM sodium dihydrogen phosphate in the iron superfusate. For the control superfusate, the concentration of NaCl was only reduced from 120 to 115.8 mM since only tri-sodium citrate (2 mM) but not ferric ions were present in this medium.

After the synaptic transients, the input resistance, the membrane potential, and the depolarization-induced discharge of action potentials were determined to be stable for 15 minutes, iron solution of 0.4 or 2 mM was applied for 15 minutes. After removal of either 0.4 or 2 mM iron solution, the slice was reperfused with the corresponding control superfusate for 30 minutes. The membrane potential was current-clamped to the control
value when the synaptic transients, the input resistance, and the depolarization-induced
discharge of action potentials were measured.

17.9) **Presynaptic Volley and the Suppression of Synaptic Transients:**

Three different concentrations of bovine hemoglobins (0.1, 0.5, or 1 mM; Sigma),
were applied for 15 minutes after the extracellularly recorded field EPSPs were
determined to be stable for 15 minutes. After each hemoglobin application, the slice was
reperfused with ACSF for 30 minutes. The amplitude of the field EPSP was plotted
against the presynaptic volley on a graph (i.e. amplitude of the field EPSP vs amplitude of
the presynaptic volley graph).
18) **RESULTS:**

18.1) **Effects of Rat Hemoglobin on hippocampal CA1 neurons:**

Superfusion of rat hippocampal slices with 0.05 mM of rat hemoglobin for 15 minutes changed the membrane potential of the neurons from \(-68.1 \pm 1.0\) mV to \(-59.2 \pm 1.8\) mV \((n=21; \ p < 0.05)\) (Fig. 3A). The membrane potential usually returned to the control level shortly after the removal of hemoglobin. In 7 out of 21 neurons, an after-hyperpolarization was observed. This hyperpolarization lasted for 5 to 10 minutes. Rat hemoglobin also increased the input resistance and suppressed the intracellularly recorded EPSPs \((% \ of \ the \ control \ EPSP \ slope: \ 37.3 \pm 8.5%; \ n = 7; \ p < 0.05)\). Partial recoveries of the EPSPs and the input resistance were observed during the first thirty minutes after the removal of hemoglobin. In these 7 neurons, the membrane potential during the application was not current clamped to the pre-drug level when records of synaptic transients were taken.

Since it is possible that the suppression of the EPSP was due to the depolarization induced by hemoglobin, in some neurons the membrane potential was current clamped. In these cells, EPSPs were suppressed and the input resistance was enhanced \((EPSP \ slope \ in \ hemoglobin \ as \ a \ % \ of \ control: \ 36.1 \pm 8.9; \ n = 7; \ p < 0.05)\).
Figure 3. Effects of hemoglobin on the membrane potential and the input resistance of rat hippocampal CA1 neurons. Hemoglobin was applied for 15 minutes. The input resistance was monitored by injecting hyperpolarizing current pulses throughout the experiment. A) Rat hemoglobin (0.05 mM) caused a depolarization and increased the input resistance. The resting membrane potential of this neuron was -58 mV. B) Bovine hemoglobin (0.1 mM) showed similar actions on the membrane potential and the input resistance in CA1 neurons of rat hippocampal slices as rat hemoglobin. 10^{-7} M TTX was added in the superfusate in order to avoid firing of action potentials during the depolarization caused by hemoglobin. The resting membrane potential of this neuron was -69 mV.
18.2) **Effects of Bovine Hemoglobin on hippocampal CA1 neurons:**

18.2.1) The effects of hemoglobin on the evoked synaptic responses, the membrane potential, the input resistance, and the excitability of CA1 neurons.

The effects of bovine hemoglobin on hippocampal CA1 neurons were studied in a total of 53 neurons with an average resting membrane potential of -65.6 ± 5.2 mV.

Different concentrations of bovine hemoglobin (0.01 - 1 mM) were used to determine the appropriate concentration for inducing significant effects consistently in CA1 neurons (n = 27). A concentration of 0.1 mM (applied for 15 minutes) was used to induce a 5.9 ± 0.9 mV (n = 26; p < 0.05) depolarization of the neurons (Fig. 3B). As in the case of rat hemoglobin, the effect of bovine hemoglobin on the membrane potential usually disappeared 2 - 5 minutes after terminating its application. Although the membrane potential usually returned to the control value upon removal of hemoglobin, an after-hyperpolarization that lasted for approximately 15 minutes was observed in 10 out of 26 neurons. Bovine hemoglobin also increased the input resistance by 51.61 ± 5.94 % (n = 26; p < 0.05). The hemoglobin-induced increase in the input resistance is clearly shown by the increase in the slope of the current-voltage curve recorded in the presence of 0.1 mM hemoglobin (Fig. 4A). This increase in the input resistance caused by hemoglobin was found to be independent of the hemoglobin-induced depolarization (Fig. 4B). Superfusion of hippocampal slices with 0.1 mM hemoglobin for 15 minutes also produced a significant suppression of the evoked synaptic responses of CA1 neurons during application (n = 6). While a depolarization of 41.2 ± 3.6 mV and an increase of 63.4 ± 11.4 % in the input resistance were observed when 0.5 mM hemoglobin was applied for 15 minutes (n = 9; p < 0.05), a depolarization of 53.6 ± 6.2 mV and an increase of 103.8 ± 28.7 % in the input resistance were seen with a 15 minutes application of 1.0 mM
hemoglobin (n = 5; \( p < 0.05 \)). Both 0.5 mM (n = 9) and 1.0 mM (n = 5) hemoglobin completely abolished the evoked synaptic responses during application. Because 0.1 mM produced less extreme effects on the membrane potential, the input resistance, and the evoked synaptic responses than the higher doses, this concentration of hemoglobin was applied for 15 minutes in further experiments.

Upon application of 0.1 mM hemoglobin, the number of spikes evoked by rectangular depolarizing pulses increased (Fig. 4C). This increase in the depolarization-induced discharge of action potentials either partially or completely recovered 30 minutes post-drug application when recordings were terminated.
Figure 4. Effects of bovine hemoglobin on the input resistance and the excitability of rat hippocampal CA1 neurons. A) Current-voltage curves recorded during control and in the presence of hemoglobin (0.1 mM; 15 min). Note the increase in the slope of the curve for hemoglobin. B) Current-voltage curves for control and bovine hemoglobin recorded after current-clamping the membrane potential to the control value. The hemoglobin-induced depolarization was compensated by a hyperpolarizing DC current. The slope of the curve for hemoglobin is steeper than that for control. Both A and B were recorded in the same neuron. The resting membrane potential of this neuron was -68 mV. C) The effect of bovine hemoglobin on the excitability of rat hippocampal CA1 neurons. The cell excitability was monitored by the number of spikes induced by injecting a 0.3 nA depolarizing rectangular pulse (200 ms). Depolarizing pulses were injected into the neurons during control (a), immediately before the end of the 15 minutes application of hemoglobin (b), and 30 minutes after the application of hemoglobin (c). The resting membrane potential of this neuron was -64 mV.
Figure 4

A

Current (nA)

-0.25 -0.15 -0.05 0.05 0.15

Δ Hb

○ Control

Membrane Potential (mV)

-85

-75

-65

-55

-45

B

Current (nA)

-0.25 -0.15 -0.05 0.05 0.15

Δ Hb

○ Control

Membrane Potential (mV)

-85

-75

-65

-55

-45

C

a

b

c

10 mV

0.3 nA

200 ms

200 ms
Without adjusting the membrane potential to the pre-drug level, the EPSP was depressed by 0.1 mM hemoglobin (EPSP slope in hemoglobin as a % of control: 26.95 ± 5.16; n = 6; p < 0.05). In these neurons, the fast and slow IPSPs were suppressed, enhanced or not changed. These varied results may be due to the depolarization caused by the drug.

When neurons were current-clamped to pre-hemoglobin membrane potentials, the agent depressed the EPSP (slope of EPSP in hemoglobin as a % of control: 27.23 ± 3.24; n = 8; p < 0.05), the fast IPSP (response in hemoglobin as a % of control: 44.56 ± 3.66; n = 10; p < 0.05), and the slow IPSP (response in hemoglobin as a % of control: 20.24 ± 6.92; n = 4; p < 0.05) (Fig. 5A & B; 6D, E, & F). The EPSP (% of control EPSP slope: 52.65 ± 10.66; n = 8; p < 0.05) and the slow IPSP (% of control response: 41.99 ± 6.31; n = 4; p < 0.05) showed only partial recoveries when measured 30 minutes after the removal of hemoglobin (Fig. 6D & F). Unlike the EPSP and the slow IPSP, the fast IPSP (% of control response: 102.48 ± 15.17; n = 10; p > 0.05) completely recovered from the hemoglobin-induced depression (Fig. 6E).
Figure 5. Effects of bovine hemoglobin on the evoked synaptic responses of rat hippocampal CA1 neurons. Bovine hemoglobin (Hb, 0.1 mM) was applied for 15 minutes. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of hemoglobin on the EPSP. The EPSP traces were taken during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after removal of hemoglobin (c). B) The effects of hemoglobin on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after removal of hemoglobin (c). The resting membrane potential of this neuron was -69 mV.
Figure 6. Time course of the effects of bovine hemoglobin on the evoked synaptic responses of rat hippocampal CA1 neurons after current-clamping the membrane potential to the control level. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. The depolarization caused by hemoglobin was compensated by a hyperpolarizing DC current so that synaptic responses could be recorded at the control membrane potential. A) Time course of the effects of normal ACSF on the EPSP. The curve was plotted with the average data of five different neurons. B) Time course of the effects of normal ACSF on the fast IPSP. The curve was plotted with the average data of five neurons. C) Time course of the effects of normal ACSF on the slow IPSP. The curve was plotted with the average data of five neurons. D) Time course of the suppression of intracellularly recorded EPSP by bovine hemoglobin (0.1 mM; 15 min). The curve was plotted with the average data of eight different neurons. E) Time course of the suppression of the fast IPSP by bovine hemoglobin (0.1 mM; 15 min). The curve was plotted with the average response of ten different neurons. F) Time course of the suppression of the slow IPSP by bovine hemoglobin (0.1 mM; 15 min). The curve was plotted with the average response of four different neurons. Slices were superfused with medium containing picrotoxinin (50 mM) throughout the experiment in order to remove the fast IPSP.
18.3) The effects of hemoglobin on the extracellularly recorded Field EPSPs:

Superfusion of slices with 0.1 (n = 3), 0.5 (n = 3), and 1.0 mM (n = 3) bovine hemoglobin for 15 minutes induced paroxysmal depolarization shifts as well as dose-dependently suppressed the extracellularly recorded field EPSPs and the presynaptic volley. At 0.1 and 0.5 mM hemoglobin, the field EPSPs started to recover at 40 and 50 minutes post-drug application, respectively. At 1.0 mM bovine hemoglobin, the field EPSPs did not start to recover until 60 minutes post-drug application. While the presynaptic volley was significantly suppressed by 0.5 mM hemoglobin, minimal change on the presynaptic volley was observed with 0.1 mM hemoglobin (Fig. 7A & B).
Figure 7. Effects of different concentrations of hemoglobin on the field EPSPs and the presynaptic volley. A) 0.1 mM (n = 3). B) 0.5 mM (n = 3). The size of the field EPSP (mV) was plotted against the size of the presynaptic volley (mV). Three different stimulation strengths were used in all six experiments. Records were taken during control, immediately before the end of drug application, and at 60 minutes after the removal of hemoglobin.
Figure 7A

- Control
- HB
- Recovery

Amplitude of EPSP (mV) vs. Amplitude of Presynaptic Volley (mV)
Figure 7B

- Control
- Hb
- Recovery

Amplitude of EPSP (mV) vs. Amplitude of Presynaptic Volley (mV)
18.4) Calcium and Hemoglobin-Induced Depolarization:

In a Ca\(^{2+}\)-free medium, the depolarization induced by hemoglobin (0.1 mM; 15 min) was enhanced significantly (\% of control response: \(223.50 \pm 28.31\); \(n = 10\); \(p < 0.05\)) (Fig. 8A & B). By 30 minutes after the replacement of the Ca\(^{2+}\)-free medium with the control medium, the enhancement in the hemoglobin-induced depolarization was reversed (\% of control response: \(102.54 \pm 2.56\); \(n = 10\); \(p > 0.05\)) (Fig 8A & B).
Figure 8. Calcium and the hemoglobin-induced depolarization. Depolarizations induced by 0.1 mM hemoglobin prepared in a normal medium containing 10^{-7} M TTX were compared to that prepared in a calcium-free medium which also contained 10^{-7} M TTX. A) A histogram was constructed with the average response of ten different neurons. B) Raw traces recorded in a neuron whose resting membrane potential was -59 mV.
Figure 8

A

% of Control Hemoglobin-Induced Depolarization

0 50 100 150 200 250

Control

Ca-free

Recovery

n = 10

B

Hb

Ca-free medium

Hb + Ca-free medium

a

10 min.

b

20 mV
18.5) Involvements of NMDA and Non-NMDA Receptors in the Hemoglobin-Induced Depolarization:

18.5.1) APV and Hemoglobin-Induced Depolarization:

In the presence of 50 μM APV, the depolarization induced by 0.1 mM hemoglobin (15 min) was decreased (% of control response: 78.25 ± 12.45; n = 10) (Fig. 9A & B). However, this APV-mediated suppression was statistically insignificant (p > 0.05). When hemoglobin was applied at 30 minutes after the removal of APV, the response recovered (% of control response 89.54 ± 6.75; n = 10; p > 0.05) (Fig. 9A & B).

18.5.2) CNQX and Hemoglobin-Induced Depolarization:

Superfusion of slices with a medium containing 20 μM CNQX enhanced the depolarization evoked by 0.1 mM hemoglobin (15 min) (% of control response: 120.14 ± 17.01; n = 6) (Fig. 10A & B). Similar to the effect of APV, the enhancement in the hemoglobin-induced depolarization caused by CNQX was statistically insignificant (p > 0.05). The effect of CNQX was reversed by 30 minutes after the removal of CNQX (% of control response: 104.01 ± 11.27; n = 6; p > 0.05) (Fig. 10A & B).
Figure 9. Effects of APV on the depolarizing action of hemoglobin. Depolarizations induced by 0.1 mM hemoglobin (15 min) were evoked in a normal medium containing $10^{-7}$ M TTX as well as a Mg$^{2+}$-free medium containing $10^{-7}$ M TTX and 50 µM APV. A) A histogram was constructed with the average response of 10 different neurons. B) Raw traces recorded in a neuron whose resting membrane potential was -69 mV.
Figure 9

A

Hb

APV

Hb

APV

Recovery

% of Control Hemoglobin-Induced Depolarization

0 20 40 60 80 100 120

Control

APV

Recovery

n = 10

10 min.

20 mV
Figure 10. Effects of CNQX on the hemoglobin-induced depolarization. Depolarizations induced by 0.1 mM hemoglobin (15 min) were evoked in a normal medium containing 10⁻⁷ M TTX as well as a medium containing 10⁻⁷ M TTX and 20 µM CNQX. A) A histogram was constructed with the average response of 6 different neurons. B) Raw traces recorded in a neuron whose resting membrane potential was -72 mV.
18.6) *Effects of Hemoglobin on the Glutamate-Induced Depolarization:*

When 5 mM glutamate was applied at the peak of the depolarization induced by hemoglobin (0.1 mM; 15 min), the glutamate response was suppressed (% of the control response: 53.62 ± 9.51; n = 8; p < 0.05) (Fig. 11A & B). When 5 mM glutamate was applied at 5, 30, and 60 minutes after the removal of hemoglobin, the glutamate-mediated response was 83.81 ± 13.41 %, 82.22 ± 21.46 %, and 95.01 ± 25.56% of the control response, respectively (n = 8; p > 0.05).
Figure 11. Effects of hemoglobin on the glutamate-induced depolarization. Depolarizations induced by 5 mM glutamate were evoked in normal ACSF and at the peak of the hemoglobin-mediated depolarization. A) A histogram was constructed with the average response of 8 different neurons. B) Raw traces recorded in a neuron whose resting membrane potential was -61 mV.
18.7) Effects of $N\omega$-nitro-L-arginine on the Actions of Hemoglobin:

18.7.1) Low Dose (100 $\mu$M) of $N\omega$-nitro-L-arginine:

Superfusion of slices with 100 $\mu$M $N\omega$-nitro-L-arginine for one hour caused a hyperpolarization of $3.20 \pm 1.07$ mV ($n = 5; \rho < 0.05$). The NO synthase inhibitor also caused a statistically insignificant increase in the input resistance (change in the input resistance as a % of control: $4.48 \pm 8.91$ % ($n = 5; \rho > 0.05$). Superfusion of hippocampal slices with 100 $\mu$M of $N\omega$-nitro-L-arginine for 1 hour produced a suppression of the EPSP (% of the control slope: $56.67 \pm 9.17$; $n = 5; \rho < 0.05$), the fast IPSP (% of the control response: $76.79 \pm 7.51$; $n = 5; \rho > 0.05$), and the slow IPSP (% of the control response: $62.11 \pm 5.66$; $n = 5; \rho < 0.05$) of CA1 neurons at the end of the application (Fig. 12A & B; 15 A, B, & C). The number of spikes evoked by rectangular depolarizing pulses was found to be increased at the end of the one hour application of $N\omega$-nitro-L-arginine (Fig. 14A & B).

When 0.1 mM hemoglobin (15 min) was applied after an hour of superfusion with 100 $\mu$M $N\omega$-nitro-L-arginine, the EPSP (slope of EPSP in hemoglobin as a % of control: $35.94 \pm 9.10$, $n = 5; \rho < 0.05$), the fast IPSP (response in hemoglobin as a % of control: $41.22 \pm 15.40$, $n = 5; \rho < 0.05$), and the slow IPSP (response in hemoglobin as a % of control: $34.07 \pm 9.69$, $n = 5; \rho < 0.05$) were depressed at the end of the hemoglobin application (Fig. 13A & B; 15D, E, & F). The EPSP (% of control EPSP slope: $65.91 \pm 15.08$, $n=5; \rho > 0.05$), the fast IPSP (% of control response: $99.83 \pm 17.16$, $n = 5; \rho > 0.05$), and the slow IPSP (% of control response: $88.03 \pm 16.95$, $n = 5; \rho > 0.05$) recovered from the hemoglobin-induced suppression at 30 minutes after the removal of hemoglobin (Fig. 13A & B; 15D, E, & F).
In addition to the suppression of the evoked synaptic responses, hemoglobin also caused a depolarization of $7.40 \pm 1.57 \text{ mV (p < 0.05)}$ and a $91.51 \pm 18.59 \% (p < 0.05)$ increase in the input resistance. An increase in the number of depolarization-induced discharge of action potentials was observed during Nω-nitro-L-arginine and hemoglobin applications (Fig. 14A, B, & C). The increase in the number of depolarization evoked action potentials was still present at 30 minutes after the removal of hemoglobin (Fig. 14D).
Figure 12. Effects of Nω-nitro-L-arginine on the evoked synaptic responses of rat hippocampal CA1 neurons. Nω-nitro-L-arginine (100 µM) was applied for 1 hour. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of Nω-nitro-L-arginine on the EPSP. The EPSP traces were taken during control (a), and immediately before the end of the application of Nω-nitro-L-arginine (b). B) The effects of Nω-nitro-L-arginine on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of Nω-nitro-L-arginine (b). The resting membrane potential of this neuron was -69 mV.
**Figure 13. Effects of hemoglobin on the evoked synaptic responses of rat hippocampal CA1 neurons in the presence of 100 μM NO-nitro-L-arginine.**

Hemoglobin (0.1 mM) was applied for 15 minutes. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of hemoglobin on the EPSP. The EPSP traces were taken during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after the removal of hemoglobin (c).

B) The effects of hemoglobin on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after the removal of hemoglobin (c). The resting membrane potential of this neuron was -69 mV.
Figure 14. The effect of hemoglobin on the excitability of rat hippocampal CA1 neurons in the presence of 100 μM Nω-nitro-L-arginine. The cell excitability was monitored by the number of spikes induced by injecting a 0.3 nA depolarizing rectangular pulse (200 ms). A depolarizing pulse was injected into the neurons during control (a), immediately before the end of the 1 hour application of hemoglobin (b), immediately before the end of the 15 minutes application of hemoglobin (c), and 30 minutes after the application of hemoglobin (d). The resting membrane potential of this neuron was -64 mV.
Figure 15. Actions of hemoglobin (0.1 mM) and Nω-nitro-L-arginine (100 μM) on evoked synaptic responses. A) Time course of the Nω-nitro-L-arginine-induced suppression of the EPSP. B) Time course of the Nω-nitro-L-arginine-induced suppression of the fast IPSP. C) Time course of the Nω-nitro-L-arginine-induced suppression of the slow IPSP. D) Time course of the suppression of intracellularly recorded EPSP by hemoglobin (0.1 mM; 15 min) in the presence of 100 μM Nω-nitro-L-arginine. E) Time course of the suppression of the fast IPSP by hemoglobin in the presence of 100 μM Nω-nitro-L-arginine. F) Time course of the suppression of the slow IPSP by hemoglobin in the presence of 100 μM Nω-nitro-L-arginine. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. The depolarization caused by hemoglobin was compensated by a hyperpolarizing DC current so that synaptic responses could be recorded at the control membrane potential. The curves were constructed with the average response of five different neurons.
Figure 15

% of Control EPSP Slope

% of Control Fast IPSP Amplitude

% of Control Slow IPSP Amplitude
18.7.2) High Dose (500 μM) of Nω-nitro-L-arginine:

Superfusion of slices with 500 μM Nω-nitro-L-arginine for one hour caused a 6.33 ± 1.02 mV (n = 6; p < 0.05) hyperpolarization. The NO synthase inhibitor also increased the input resistance by 36.73 ± 13.02 % (n = 6; p < 0.05). Superfusion of hippocampal slices with 500 μM Nω-nitro-L-arginine for 1 hour produced a suppression of the EPSP (% of the control slope: 77.02 ± 10.03; n = 6), and an enhancement of the fast IPSP (% of the control response: 173.18 ± 33.18; n = 6) as well as the slow IPSP (% of the control response: 126.70 ± 14.90; n = 6) of CA1 neurons at the end of the application (Fig. 16A & B; 19 A, B, & C). However, the Nω-nitro-L-arginine-induced changes in the EPSP, the fast IPSP, and the slow IPSP were statistically insignificant (p > 0.05). The number of spikes evoked by rectangular depolarizing pulses was increased at the end of the one hour application of Nω-nitro-L-arginine (Fig. 18A & B).

When 0.1 mM hemoglobin (15 min) was applied after an hour of superfusion with 500 μM Nω-nitro-L-arginine, the EPSP (slope of EPSP in hemoglobin as a % of control: 27.46 ± 10.06, n = 6; p < 0.05), the fast IPSP (response in hemoglobin as a % of control: 53.60 ± 16.54, n = 6; p < 0.05), and the slow IPSP (response in hemoglobin as a % of control: 49.05 ± 13.00, n = 6; p < 0.05) were depressed (Fig. 17A & B; 19D, E, & F). The EPSP (% of control EPSP slope: 70.38 ± 1.05, n = 6; p > 0.05), the fast IPSP (% of control response: 94.81 ± 12.66, n = 6; p > 0.05), and the slow IPSP (% of control response: 110.54 ± 5.32, n = 6; p > 0.05) recovered from the hemoglobin-induced suppression at 30 minutes after the removal of hemoglobin (Fig. 17A & B; 19D, E, & F).

Besides the changes in the evoked synaptic responses, hemoglobin also caused a depolarization of 11.00 ± 3.42 mV (p < 0.05) and a 72.13 ± 6.20 % (p <0.05) increase in the input resistance. An increase in the number of depolarization-induced discharge of
action potentials was observed during and at 30 minutes after hemoglobin application (Fig. 18C & D).
Figure 16. Effects of \( N_\omega \)-nitro-L-arginine (500 \( \mu \)M) on the evoked synaptic responses of rat hippocampal CA1 neurons. \( N_\omega \)-nitro-L-arginine (500 \( \mu \)M) was applied for 1 hour. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of \( N_\omega \)-nitro-L-arginine on the EPSP. The EPSP traces were taken during control (a), and immediately before the end of the application of \( N_\omega \)-nitro-L-arginine (b). B) The effects of \( N_\omega \)-nitro-L-arginine on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of \( N_\omega \)-nitro-L-arginine (b). The resting membrane potential of this neuron was -58 mV.
Figure 17. Effects of hemoglobin on the evoked synaptic responses of rat hippocampal CA1 neurons in the presence of 500 μM Nω-nitro-L-arginine. Hemoglobin (0.1 mM) was applied for 15 minutes. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. The resting membrane potential of this neuron was -65 mV.

A) The effects of hemoglobin on the EPSP. The EPSP traces were taken during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after the removal of hemoglobin (c).

B) The effects of hemoglobin on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of hemoglobin (b), and at 30 minutes after the removal of hemoglobin (c).
Figure 18. The effect of hemoglobin on the excitability of rat hippocampal CA1 neurons in the presence of 500 μM No-nitro-L-arginine. The cell excitability was monitored by the number of spikes induced by injecting a 0.1 nA depolarizing rectangular pulse (200 ms). A depolarizing pulse was injected into the neurons during control (a), immediately before the end of the 1 hour application of hemoglobin (b), immediately before the end of the 15 minutes application of hemoglobin (c), and 30 minutes after the application of hemoglobin (d). The resting membrane potential of this neuron was -60 mV.
Figure 19. Actions of hemoglobin (0.1 mM) and Nω-nitro-L-arginine (500 μM). A) Time course of the Nω-nitro-L-arginine-induced suppression of the EPSP. B) Time course of the Nω-nitro-L-arginine-induced suppression of the fast IPSP. C) Time course of the Nω-nitro-L-arginine-induced suppression of the slow IPSP. D) Time course of the suppression of intracellularly recorded EPSP by hemoglobin (0.1 mM; 15 min) in the presence of 500 μM Nω-nitro-L-arginine. E) Time course of the suppression of the fast IPSP by hemoglobin in the presence of 500 μM Nω-nitro-L-arginine. F) Time course of the suppression of the slow IPSP by hemoglobin in the presence of 500 μM Nω-nitro-L-arginine. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. The depolarization caused by hemoglobin was compensated by a hyperpolarizing DC current so that synaptic responses could be recorded at the control membrane potential. The curves were constructed with the average response of six different neurons.
Figure 19

A: % of Control EPSP Slope

B: % of Control Fast IPSP Amplitude

C: % of Control Slow IPSP Amplitude

D: % of Control EPSP Slope

E: % of Control Fast IPSP Amplitude

F: % of Control Slow IPSP Amplitude
18.8) **Iron and the Actions of Hemoglobin:**

18.8.1) **Low Dose (0.4 mM) of Ferric Chloride:**

Superfusion of slices with 0.4 mM ferric chloride for 15 minutes produced a suppression of the EPSP (% of the control slope: 80.94 ± 4.62; n = 8), the fast IPSP (% of the control response: 90.16 ± 6.84; n = 8), and the slow IPSP (% of the control response: 77.90 ± 6.01; n = 7) when measured at the end of the application (Fig. 20A & B; 24A, B, & C). However, the iron-induced changes in the evoked synaptic responses were statistically insignificant (p > 0.05). At 30 minutes after the removal of iron, the EPSP (% of the control slope: 83.05 ± 10.76; n = 8), the fast IPSP (% of the control response: 80.30 ± 10.24; n = 8), and the slow IPSP (% of the control response: 78.77 ± 8.39; n = 7) were not significantly different (p > 0.05) from those recorded at the end of the iron application (Fig. 20A & B; 24A, B, & C). The membrane potential, the input resistance, and the number of depolarization-induced discharge of action potentials, of the neurons were not changed during iron application (Fig. 21).
Figure 20. Effects of 0.4 mM ferric chloride on the evoked synaptic responses of rat hippocampal CA1 neurons. Ferric chloride application was -57 mV. The neuron was -63 mV.

The resting membrane potential of this neuron was -63 mV. Ferric chloride (0.4 mM) was applied for 15 minutes. The synaptic responses were recorded during control (A). Immediately before the end of the application of ferric chloride, the synaptic responses were recorded (B), and at 30 minutes after the removal of ferric chloride, the synaptic responses were recorded again (C). The effects of ferric chloride on the evoked synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of ferric chloride on the EPSP. B) The effects of ferric chloride on the fast and slow IPSPs.
Figure 21. The effect of 0.4 mM ferric chloride on the excitability of rat hippocampal CA1 neurons. The cell excitability was monitored by the number of spikes induced by injecting a 0.2 nA depolarizing rectangular pulse (200 ms). A depolarizing pulse was injected into the neurons during control (a), immediately before the end of the 15 minutes application of ferric chloride (b), and 30 minutes after the application of ferric chloride (c). The resting membrane potential of this neuron was -63 mV.
18.8.2) High Dose (2.0 mM) of Ferric Chloride:

After superfusing slices with 2.0 mM of ferric chloride for 15 minutes, while the EPSP (% of the control slope: 117.55 ± 12.69; n = 5; p < 0.05) was enhanced, the fast IPSP (% of the control response: 100.63 ± 16.10; n = 8; p > 0.05), and the slow IPSP (% of the control response: 90.57 ± 11.56; n = 7; p > 0.05) were not altered significantly when measured at the end of the application (Fig. 24D, E, & F). At 30 minutes after the removal of iron, the EPSP (% of the control slope: 92.21 ± 7.22; n = 8; p > 0.05) recovered. The fast IPSP (% of the control response: 74.56 ± 9.54; n = 8), and the slow IPSP (% of the control response: 78.33 ± 9.00; n = 7) recorded at 30 minutes post-iron application were not significantly changed (p > 0.05) compared to the measurements recorded at the end of the iron application (Fig. 22A & B; 24D, E, & F). Like the low dose of ferric chloride, the high dose of ferric chloride also did not change the membrane potential. The number of depolarization-induced discharge of action potentials was marginally suppressed in the presence of 2.0 mM ferric chloride (Fig. 23). Even though the input resistance was not altered by 0.4 mM of ferric chloride, a decrease of 12.23 ± 1.52 % (p < 0.05) in the input resistance was caused by 2.0 mM ferric chloride.
Figure 22. Effects of 2.0 mM ferric chloride on the evoked synaptic responses of rat hippocampal CA1 neurons. Ferric chloride (2.0 mM) was applied for 15 minutes. The synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum. A) The effects of ferric chloride on the EPSP. The EPSP traces were taken during control (a), immediately before the end of the application of ferric chloride (b), and at 30 minutes after the removal of ferric chloride (c). B) The effects of ferric chloride on both fast and slow IPSPs. The synaptic responses were recorded during control (a), immediately before the end of the application of ferric chloride (b), and at 30 minutes after the removal of ferric chloride (c). The resting membrane potential of this neuron was -62 mV.
Figure 23. The effect of 2.0 mM ferric chloride on the excitability of rat hippocampal CA1 neurons. The cell excitability was monitored by the number of spikes induced by injecting a 0.2 nA depolarizing rectangular pulse (200 ms). A depolarizing pulse was injected into the neurons during control (a), immediately before the end of the application of ferric chloride (b), and 30 minutes after the application of ferric chloride (c). The resting membrane potential of this neuron was -68 mV.
Figure 24. Time course of the effects of ferric chloride on evoked synaptic responses were recorded in response to 0.05 Hz stimulation of the stratum radiatum.
**19) DISCUSSION:**

In hemorrhagic stroke and other cerebrovascular injuries, blood often accumulates intracranially for a considerable period of time (Somás et al., 1972). Slow hemolysis of erythrocytes with release of hemoglobin into the supernatant fluid has been reported to occur after 2 days of in-vitro incubation of blood (Asano et al., 1980; Barrows et al., 1955; Osaka, 1977; Miyaoka et al., 1976; Sonobe and Suzuki, 1978; Sasaki et al., 1979; Okwuasaba, 1981; Duff et al., 1987). After intracranial bleeding or hemorrhagic stroke, erythrocytes, which can remain in the intracranial cavity for days, are hemolysed in a similar fashion as in in-vitro incubation (Barrows et al., 1955; Findlay et al., 1989). After 2 hours of subarachnoid hemorrhage, hemoglobin was released from erythrocytes (Barrows et al., 1955). Because hemoglobin can remain in the cerebrospinal fluid for weeks (Barrows et al., 1955), neurons may be exposed to hemoglobin. In fact, hemoglobin has been suggested to induce cerebral vasospasm associated with subarachnoid hemorrhage (Weir, 1987; Osaka et al., 1980). Moreover, its iron content may be related to stroke- or head injury-induced epilepsy (Hammond et al., 1980). To date, little is known about the electrophysiological actions of hemoglobin. Therefore, in the present investigation, we examined the actions of hemoglobin on the membrane potential, the input resistance, the ability to discharge action potentials, and the evoked synaptic responses, of CA1 pyramidal neurons in rat hippocampal slices.

In the present study, hemoglobin was found to induce a depolarization, to increase the input resistance and the depolarization-induced discharge of action potentials, as well as to suppress the EPSP and IPSPs of the CA1 neurons.
19.1) **Suppressions of the Evoked Synaptic Responses:**

The hemoglobin-induced suppression of the EPSP, which might attenuate the network activity by decreasing inter-neuronal communications, did not seem to be caused by the depolarization evoked by the drug since current-clamping the membrane potential to the pre-hemoglobin level did not abolish the suppression of the EPSP. Because hemoglobin significantly suppressed the glutamate-evoked depolarization even after the hemoglobin-induced depolarization was compensated with a current clamp, it is possible that hemoglobin suppressed the EPSP by interfering with excitatory transmission. The suppression of the actions of glutamate may be due to blockade of the excitatory amino acid receptors or the calcium influx at postsynaptic terminals. However, the suppression of the EPSP may be due to a number of other reasons unrelated to an action at the amino acid receptor sites as hemoglobin-induced suppression of the EPSP is larger than that of the glutamate-induced depolarization. The difference between the time taken for recovery of the glutamate-induced depolarization and that of the EPSPs from hemoglobin-induced depression supports the idea that the interference with the excitatory transmission is not the sole cause of the suppression of the EPSP. An inhibition of the propagation of action potentials in the presynaptic axons could also be a cause of the EPSP suppression since EPSPs and IPSPs were both suppressed by hemoglobin. Hemoglobin (0.1 mM or 0.5 mM) suppressed the presynaptic volley. While the suppression caused by 0.1 mM hemoglobin is minimal, that induced by 0.5 mM hemoglobin is significant. These observations suggest that the suppression of the EPSP induced by 0.1 mM hemoglobin may be partly attributed to an interference with the propagation of presynaptic action potentials.
In this study, the mechanisms which underlie the suppressions of both fast and slow IPSPs were not explored. These actions of hemoglobin may be due to a suppression of the GABA-ergic inhibition or GABA release. Hemoglobin may act at its own receptors. If an interaction at the receptor level is ruled out, effects of blocking second messenger systems such as the inositol phosphate/Ca$^{2+}$ system on the actions of hemoglobin should be examined.

19.2) **Actions of Hemoglobin on the Membrane Potential, the Input Resistance, and the Depolarization-Induced Discharge of Action Potentials:**

The depolarizing action of hemoglobin probably was not caused by activations of either NMDA or non-NMDA glutamate receptors and was not secondary to the release of glutamate since this action of hemoglobin was insignificantly altered in the presence of APV nor CNQX but was present in a Ca$^{2+}$-free and TTX-containing medium. In addition, the hemoglobin-induced depolarization probably does not require a Ca$^{2+}$-influx because an exclusion of extracellular calcium ions did not suppress the depolarization. In the calcium-free medium, the enhancement in the depolarizing action of hemoglobin when applied in the control medium is not entirely understood but may suggest that the agent may actually enhance a calcium-activated potassium current. During the hemoglobin-induced depolarization, calcium-activated potassium channels may be activated by calcium entering through the voltage-dependent calcium channels. As a consequence of the hyperpolarization caused by potassium entering through the calcium-activated potassium channels, the depolarizing action of hemoglobin is dampened in a calcium-containing medium.

Because the slow IPSP was suppressed by hemoglobin, it is possible that closures of K$^+$ channels may partially underlie the depolarization, the increase in the input
resistance, and the loss of spike frequency adaptation during depolarization caused by hemoglobin. In hippocampal pyramidal cells, activation of muscarinic receptor is known to decrease the leak $K^+$ current, the $Ca^{2+}$-activated $K^+$ current, the noninactivating voltage-dependent $K^+$ current ($I_{M}$), and the transient voltage-dependent $K^+$ current ($I_{A}$). Application of acetylcholine and carbachol have been reported to strongly depolarize hippocampal pyramidal cells, to increase the input resistance, and to suppress the spike frequency adaptation during depolarizing pulses (Bernado and Prince, 1982; Cole and Nicoll, 1983; Cole and Nicoll, 1984a; Cole and Nicoll, 1984b). While a decrease in the voltage-independent leak $K^+$ current has been suggested to underlie the depolarization caused by muscarinic receptors activation, a blockade of the $Ca^{2+}$-activated $K^+$ current could lead to a loss of spike frequency adaptation during a depolarizing pulse (Bernado and Prince, 1982; Benson et al., 1988; Madison et al., 1987b). Because muscarinic receptors activation and hemoglobin have similar actions on the membrane potential, the input resistance, and the spike frequency adaptation during a depolarizing pulse, it is possible that hemoglobin acts either on muscarinic receptors or blocks the same ion channels via an unknown mechanism. The use of muscarinic antagonists will be useful in determining if hemoglobin functions at the muscarinic receptor level. If hemoglobin does not interact with muscarinic receptors, effects of a non-specific blockade of $K^+$ channels by intracellular $Cs^+$ on the depolarization, the increase in input resistance, and the spike frequency adaptation during depolarizing pulses mediated by hemoglobin should be examined. It is expected that this nonspecific blockade of $K^+$ channels will shed some light on the relationship between closures of $K^+$ channels and the actions of hemoglobin on the membrane potential, the input resistance, and spike frequency adaptation during a depolarizing pulse. In the present study, however, it is unclear whether the depolarization
induced by hemoglobin is due to the increase in the input resistance as no attempt was made to correlate these two actions of hemoglobin.

Besides the closures of potassium channels, closures of chloride channels could also be a contributing factor to the hemoglobin-induced increase in the input resistance since the fast IPSP was suppressed by hemoglobin. However, this could not be the sole cause of the hemoglobin-induced depolarization and increase in the input resistance since the suppression of fast IPSP has a later onset than the depolarization and the increase in input resistance caused by hemoglobin.

19.3) Possible Involvement of Metabotropic Receptors in the Actions of Hemoglobin:

In the mammalian central nervous system, trans-ACPD, a selective agonist for metabotropic glutamate receptors, has been suggested to cause depolarization of thalamic (Hall et al., 1979) and spinal cord (McLennan et al., 1982; McLennan and Liu, 1982) neurons, to induced membrane potential oscillations in neurons of rat dorsolateral septal nucleus (Zheng and Gallagher, 1991), to decrease EPSPs in the striatum (Lovingier, 1991), and to induce calcium mobilization in cultured cerebellar neurons (Irving et al., 1990). In hippocampal CA1 area, trans-ACPD has been reported to decrease evoked EPSPs at the Schaffer collateral-CA1 pyramidal cell synapse (Baskys and Malenka, 1991a, b), to block synaptic inhibition (Desai and Conn, 1991), and to have direct excitatory effects on pyramidal cells (Desai and Conn, 1991; Stratton et al., 1989). The direct excitatory effects include pyramidal cell depolarization accompanied by an increase in input resistance, blockade of spike frequency adaptation, and inhibition of a slow AHP that follows a burst of action potentials (Desai and Conn, 1991; Stratton et al.,
1989). Not only are these effects observed in CA1, but also in CA3 (Charpak et al., 1990).

Even though actions of moderate concentrations of trans-ACPD are often assumed to be mediated by the activation of the phosphoinositide hydrolysis-linked glutamate receptor, recent cloning of multiple metabotropic glutamate receptor subtypes indicates that some of the actions of trans-ACPD may not be mediated via the activation of the phosphoinositide hydrolysis-linked glutamate receptor (Tanabe et al., 1992). In area CA1 of the hippocampus, the effects of trans-ACPD have been suggested to be mediated by a metabotropic glutamate receptor which is not phosphoinositide hydrolysis-linked because AP3, which effectively inhibits trans-ACPD-induced phosphoinositide hydrolysis, does not block these effects (Desai et al., 1992).

Activation of metabotropic receptors has been reported to block a voltage-sensitive potassium current which is known as an inward rectifier (Nicoll et al., 1990) as well as a non-inactivating and voltage-dependent potassium current \( (I_M) \) (Brown, 1990). While the blockade of the inward rectifier could increase in the input resistance (Nicoll et al., 1990), the inhibition of \( I_M \) may contribute to the membrane depolarization as well as reduced accommodation. In addition to potassium conductances, activation of metabotropic receptors also depresses calcium currents (Lester and Jahr, 1990). This action of metabotropic receptors can lead to a decrease in calcium influx at the presynaptic terminal and therefore a decrease in neurotransmitter release (Lester and Jahr, 1990). Indeed, a presynaptic rather than postsynaptic mechanism has been suggested to underlie the suppressions of glutamate and GABA-mediated synaptic potentials in striatal neurons (Calabresi et al., 1992) and suppressions of evoked field potentials in the CA1-Schaffer collateral pathway (Baskys and Malenka, 1991a, b) mediated by metabotropic
receptors. For this reason, it is possible that suppressions of both EPSPs and IPSPs reported by Desai et al. (1992) are also mediated through a decrease in calcium influx at the presynaptic terminal.

In comparison to the effects of hemoglobin, the actions of trans-ACPD except the blockade of AHP are almost identical to those of hemoglobin. For this reason, the electrophysiological actions of hemoglobin may be caused by activation of a subtype of metabotropic receptors which is not linked to phosphoinositide hydrolysis or activation of the same second messenger system as this metabotropic receptor. However, the link between activation of this metabotropic receptor subtype and the reported actions is yet to be determined because no specific antagonist is available for this subtype of metabotropic receptors. Moreover, it appears that the depolarization induced by hemoglobin is not due to a presynaptic action as it is observed in a Ca^{2+}-free and TTX-containing medium; and the depolarization induced by applied glutamate was suppressed by hemoglobin presumably through a postsynaptic mechanism of action.

19.4) Implications of the Actions of Hemoglobin:

When the GABA-mediated inhibitions are depressed, the non-NMDA receptor-mediated EPSP is prolonged (Collingridge and Lester, 1989). Therefore, NMDA receptors will be activated as a result of the prolonged depolarization (Collingridge and Lester, 1989). Because the NMDA receptor-mediated EPSP has a long duration and increases in size with membrane depolarization, it may promote repetitive firing and therefore contributing to abnormal activity at an epileptic focus (Collingridge and Lester, 1989). Moreover, agents that suppress GABA-ergic IPSPs can induce epileptiform activity. For these reasons, the hemoglobin-induced suppressions of both fast and slow IPSPs may lead to an increase in the excitability of CA1 neurons.
Even though the underlying mechanisms of the depolarizing action of hemoglobin are not clear, it may have important implications. The hemoglobin-induced depolarization could open voltage-dependent calcium channels and therefore leading to neuronal damage as a result of excessive influx of calcium into neurons. In addition, this depolarization may remove voltage-dependent Mg$^{2+}$ blockade of NMDA channels and thus causing excitotoxicity by an excessive influx of sodium and calcium ions through NMDA channels (Choi, 1985 and 1987; Choi et al., 1988; Garthwaite and Garthwaite, 1986; Garthwaite et al., 1986). In order to determine whether excessive calcium influx occurs during hemoglobin-induced depolarization, Fura-2 dye can be used to trace the mobilization of calcium during the hemoglobin-induced depolarization. If excessive calcium influx actually occurs, the controversial role of NO in excitotoxicity may be explained since hemoglobin may play a protective role by chelating NO and an offensive role by increasing calcium entry. The net effect of hemoglobin will then rely on the balance between the NO scavenging property and the depolarizing action of hemoglobin.

Similar to the depolarizing action of hemoglobin, the hemoglobin-induced increase in the input resistance may also cause an increase in the excitability of neurons since an increase in the input resistance can lead to an increase in the depolarization-induced discharge of action potentials which may be epileptogenic. The paroxysmal depolarization shifts observed after the application of hemoglobin during extracellular recordings suggest that neurons are prone to seizures after exposure to hemoglobin.

19.5) Nitric Oxide (NO) Scavenging Property of Hemoglobin:

Since 0.1 mM hemoglobin has been suggested to block LTP and excitotoxicity by scavenging NO (Schuman and Madison, 1991), it is possible that the actions of hemoglobin on the CA1 hippocampal neurons are also due to chelation of NO. In the
current investigation, however, neither 100 μM, an effective concentration in blocking LTP (Schuman and Madison, 1991), nor 500 μM Nω-nitro-L-arginine was capable of removing the hemoglobin-induced suppression of the evoked synaptic potentials. The depolarization, the increase in the input resistance, and the increase in the depolarization-induced discharge of action potentials caused by hemoglobin in the presence of the NO synthase inhibitor (100 μM or 500 μM) indicate that the reported actions of hemoglobin are mostly independent of its NO scavenging property.

19.6) The Iron Component of Hemoglobin:

In traumatic brain injuries, seizures as well as deposition of iron derived from hemoglobin in neurons have been reported (Caveness, 1963; Reid et al., 1979; Willmore et al., 1978a; Willmore et al., 1978b). Since each molecule of hemoglobin contains 4 ferrous ions, it is possible that iron accumulates near the neurons and glia after hemoglobin is broken down intracranially. Even though hemoglobin is normally metabolized in the liver, spleen, and kidney, it is possible that under physiological conditions hemoglobin is broken down as a result of the ischemia-induced change in pH of the intracranial cavity. For these reasons, it is important to examine the actions of iron on hippocampal CA1 neurons. The effects of iron on hippocampal CA1 neurons were compared to those of hemoglobin. The statistically insignificant changes in the evoked synaptic responses caused by 0.4 mM or 2.0 mM of ferric chloride suggest that the actions of hemoglobin are not due to its iron content. Similarly, the depolarization, the increase in the input resistance, and the increase in the depolarization-induced discharge of action potentials caused by hemoglobin are unlikely to be mediated by its iron content because these parameters were not altered significantly by neither 0.4 mM nor 2.0 mM ferric chloride. However, ferrous ion may have different actions on CA1 neurons and their
actions should be determined before entirely ruling out the involvement of iron in the actions of hemoglobin.

19.7) Future Studies Required for Determining the Role of Hemoglobin in Neurological Deficits Induced by Cerebrovascular Injuries:

In the present investigation, superfusion solution which contains hemoglobin was oxygenated with carbogen (95% O₂ 5% CO₂). However, in hemorrhagic stroke and in cerebrovascular injuries, oxygen supply to neurons could be compromised and therefore leading to hypoxia. For this reason, future experiments which examine the actions of hemoglobin in ischemia are necessary for further understanding of hemoglobin's role in hemorrhagic stroke and head injuries mediated neurological disorders. It is known in the literature that ischemia induces an anoxic depolarization followed by a persistent depolarization (Garaschuk et al., 1993; Rader and Lanthom, 1989). While the anoxic depolarization is seen during ischemia, the persistent depolarization is present during the early stage of reperfusion. In order to examine the actions of hemoglobin in ischemia, hemoglobin should be applied during ischemia, but not in reperfusion, during reperfusion but not in ischemia, and during both ischemia and reperfusion so that the actions of hemoglobin could be isolated from those induced by ischemia.

In addition to ischemia, the actions of heme and globulin on CA1 hippocampal neurons should also be examined. These experiments will shed some light on the mechanisms of action of hemoglobin on hippocampal CA1 neurons.
20) CONCLUSION:

Although hemoglobin has been suggested to block LTP (Böhme et al., 1991; Haley et al., 1992; Musleh et al., 1993; O'Dell et al.; 1991; Schuman and Madison, 1991) and to suppress excitotoxicity (Izumi et al., 1992) in literature, the direct electrophysiological effects of hemoglobin still have not been directly examined. In this investigation, the effects of hemoglobin on rat hippocampal CA1 neurons are shown to be broader than what have been reported in literature. Even though chelation of NO by hemoglobin has been suggested as the mechanism of LTP and excitotoxicity suppressions in literature, it is not involved in the electrophysiological actions of hemoglobin reported in this investigation. The iron content of hemoglobin also does not seem to be a contributing factor for the actions of hemoglobin. While the suppression of the EPSP could be partly attributed to the suppression of the actions of glutamate and the presynaptic volley, the mechanisms for suppressions of both fast and slow IPSPs are still unknown.

Although the underlying mechanisms of the actions of hemoglobin are still not clear, effects such as the depolarization and the increase in the input resistance have important implications. While the hemoglobin-induced depolarization, which is independent of extracellular calcium and does not interact with the excitatory amino acid receptors, may cause neuronal death by excessive calcium influx through voltage-dependent calcium or NMDA channels, the increase in the input resistance may increase the excitability of neurons.

Effects reported in this investigation such as the change in the membrane potential, the increase in the input resistance, the increase in the depolarization-induced discharge of action potentials, and suppressions of the evoked synaptic responses, of CA1 hippocampal neurons may play a role in modulating LTP and excitotoxicity. More
importantly, these actions of hemoglobin may contribute to the neurological deficits associated with cerebrovascular injuries and hemorrhagic stroke. Further studies on the underlying mechanisms of the actions of hemoglobin will allow further insight into the role of hemoglobin in neuronal damages associated with hemorrhagic stroke and head injuries.
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