SEROTONIN PARTICIPATION IN THE SUPPRESSION OF FOCAL EPILEPTIFORM ACTIVITY BY NOXIOUS STIMULATION

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PETER MAURICE THOMPSON

SEROTONIN PARTICIPATION IN THE SUPPRESSION OF FOCAL EPILEPTIFORM ACTIVITY BY NOXIOUS STIMULATION

by

© Peter Maurice Thompson, B.Sc. (Honours)

A thesis submitted in partial fulfillment
of the requirements for the degree of
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Abstract

Noxious stimulation acting, at least in part, by a serotonergic mechanism suppresses penicillin-induced focal epileptiform activity in the rat (Neuman, 1986b). In the present study the site of serotonin's action and the serotonin receptor subtype involved in the process were investigated. The working hypothesis was that an increase in the cortical concentration of serotonin arising from the rostral raphe nuclei mediated the suppression of focal epileptiform activity by noxious stimulation.

The systemic administration of agents known to inhibit raphe unit activity including serotonin agonists 8-OH-2-(di-n-propylamine)tetralin, 2-methoxyphenylpiperazine and trifluoromethylpiperazine as well as blockers of serotonin uptake, imipramine and fluoxetine, prevented the suppression of focal epileptiform activity by noxious stimulation. Analgesia at the spinal cord level was not thought to be a factor in this blockade, but rather the drugs were considered to act by inhibiting dorsal raphe neurons.

The pressure ejection of serotonin and 8-OH-2-(di-n-propylamine)tetralin into the dorsal raphe also blocked the suppression of focal epileptiform activity by noxious stimulation, despite a several minute delay between pressure ejection of drug and response. Available histology confirmed that the majority of responsive sites were located in the dorsal raphe, while the majority of non-responsive

sites were located outside the dorsal raphe region. While the involvement of the dorsal raphe was clearly established in this study, the degree of this involvement could not be determined.

Studies at the cortical level involving the pressure ejection of 1) the serotonin precursor L-5-hydroxytryptophan in rats pretreated with p-chlorophenylalanine, and 2) the serotonin releaser p-chloroamphetamine, provided some evidence that serotonin at the cortical level was essential to the suppression of focal epileptiform activity by noxious stimulation. However, similar studies with serotonin or fenfluramine failed to show any effect.

Serotonin antagonists with affinity for 5-HT_2 and 5-HT_{1C}, but not 5-HT_3 receptors, blocked the suppression of focal epileptiform activity by noxious stimulation. Based on the available data it appears that 5-HT mediates the suppression of focal epileptiform activity by noxious stimulation by an action at 5-HT_2 and/or 5-HT_{1C} receptors.

Key Words

Focal Epileptiform Activity, Serotonin, Noxious Stimulation, Dorsal Raphe, Cerebral Cortex

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Table of Contents

	Page
List of Tables	viii.
List of Figures	ix.
Chapter 1	
Introduction	
1.1 The Definition of Epilepsy	1
1.2 Neurophysiology of Seizure Generation	1
1.3 Animal Models of Epilepsy	4
1.4 Neurotransmitters and Their Role in Seizures	7
1.4.1 Gamma-aminobutyric acid (GABA)	8
1.4.2 Catecholamines	9
1.4.3 Serotonin	12
1.5 The Effect of Noxious Stimulation on Epileptiform Activity	18
1.6 Serotonergic Raphe-Cortical Projections in the Rat	19
1.7 Serotonin Receptors in the Rat CNS	19
1.7.1 Electrophysiologically characterized 5-HT receptors	22
1.7.2 Classification of 5-HT binding sites by the radioligand method	23
1.7.3 Radioligand versus physiological studies	25
1.7.4 The degree of correlation between radioligand and physiological studies	26
1.8 Rationale For the Present Study	27
Chapter 2	
Materials and Methods	
2.1 Animals	29
2.2 Surgery	29
2.2.1 Preparatory surgery	29
2.2.2 General surgery	29
2.3 Micropipettes	32
2.3.1 Intracortical recording and drug	32

delivery
2.3.2 Drug delivery to the raphe nuclei 35

2.4 Electrical Circuitry 35

2.5 Experimental protocol 35
2.5.1 Pretreatment with PCPA 36
2.5.2 I.V. injection of drugs 36
2.5.3 Intracortical pressure ejection of drugs 37
2.5.4 Local pressure ejection of drugs into the raphe nuclei 37

2.6 Statistics 38

2.7 Immunohistochemistry 38

2.8 Drugs 40

Chapter 3

Results

3.1 Noxious Stimulation and the Suppression of FEA 42

3.2 5-HT Agonists and the Suppression of FEA by NS 42

3.3 The Effect of Amine Uptake Blockers on the Suppression of FEA by NS 45
3.3.1 Desimipramine (DMI) and imipramine (IMI) and the suppression of FEA by NS 45
3.3.2 Fluoxetine (FLU) 54

3.4 The Effect of Quipazine (QPZ) on the Suppression of FEA by NS 59

3.5 The Pressure Ejection of 5-HT and 8-OH-DPAT Into the Dorsal Raphe 59
3.5.1 5-HT 66
3.5.2 8-OH-DPAT 69

3.6 Intracortical Application of L-5-hydroxytryptamine (L-5-HTP) in PCPA Treated Rats. 74
3.61 Effects of L-5-HTP on FEA 74
3.62 Immunohistochemistry 77

3.7 Intracortical Application of 5-HT and Drugs Releasing 5-HT 82
3.7.1 Intracortical pressure ejection of 5-HT 82
3.7.2 Intracortical application of 82

p-chloroamphetamine (PCA)
3.7.3 The pressure ejection of fenfluramine (FEN) 83

3.8 Baclofen and the Suppression of FEA by NS 86

3.9 Identification of the 5-HT Receptor Subtype Mediating the Suppression of FEA by NS 86

Chapter 4
Discussion

4.1 The Generation of FEA by Intracortical Pressure Ejection of Penicillin (PG) 94

4.2 The Site of Action of the Suppression of FEA by NS 95

4.3 Determination of the 5-HT receptor subtype involved in the Suppression of FEA by NS 104

4.4 Evidence for 5-HT Mediating the Suppression of FEA by NS 108

References 109

List of Tables

		Page
1.	The change in the background level of FEA and the duration of the blockade of the suppression of FEA by NS following 8-OH-DPAT, 2-MPP and TFMPP.	49
2.	The change in the background level of FEA following the administration of IMI, DMI and QPZ.	56
3.	The change in the background level of FEA and the duration of the blockade of the suppression of FEA by NS following FLU.	63
4.	The effect of QPZ on the suppression of FEA by NS in 8-OH-DPAT pretreated rats, DMI and IMI pretreated rats and untreated rats.	65
5.	The response obtained following the pressure ejection of 100 mM 5-HT at various depths in the dorsal raphe.	68
6.	The response obtained following the pressure ejection of 1 and 10 mM 8-OH-DPAT at various depths in the dorsal raphe.	71
7.	The response obtained following the pressure ejection of 100 mM 8-OH-DPAT at various depths in the dorsal raphe.	73
8.	The duration of the change of background level of FEA following the intracortical pressure ejection of L-5-HTP in PCPA pretreated rats.	79
9.	The duration of the change of background level of FEA following the intracortical pressure ejection of PCA.	85
10.	The change in background level of FEA following the i.v. administration of baclofen.	90
11.	The effect of various 5-HT antagonists on the suppression of FEA by NS.	93

List of Figures

		Page
1.	The mechanism of depletion of 5-HT by PCPA.	16
2.	The cannulation of the femoral vein of the rat using PE-50 tubing.	31
3.	The stereotaxic setup of a rat during the intracortical recording procedure	34
4.	The effect of PG pressure ejected into the rat cortex.	44
5.	The i.v. administration of 8-OH-DPAT blocks the suppression of FEA by NS in the rat.	47
6.	The i.v. administration of 2-MPP blocks the suppression of FEA by NS in the rat.	51
7.	The i.v. administration of TFMPP blocks the suppression of FEA by NS in the rat.	53
8.	The effect of DMI, IMI and QPZ on the suppression of FEA by NS in the rat.	58
9.	Fluoxetine blocks the suppression of FEA by NS in the rat.	61
10.	Histological localization of the micropipette tracks in the vicinity of the dorsal raphe.	76
11.	Immunohistochemistry performed on both PCPA treated rats and untreated rats.	81
12.	The i.v. administration of baclofen blocks the suppresion of FEA by NS in the rat.	88

Chapter 1

Introduction

1.1 The Definition of Epilepsy

Epilepsy is a disorder of brain function. The term epilepsy refers not to a single disease entity but rather to a group of disorders which have in common the clinical phenomenon of repetitive seizures. A seizure is an involuntary episodic event characterized at the cellular level by an uncontrolled synchronous firing of large aggregates of neurons (Snead, 1983). Seizures may be classified in two broad categories: 1) focal seizures and 2) generalized seizures. Focal seizures arise from a single focus, or multiple foci, in the gray matter of the cerebral cortex and either remain localized or secondarily generalize (spread throughout the brain). Generalized seizures appear to have bihemispheric involvement from their earliest manifestations. The diverse clinical manifestations and etiologies of the epilepsies seem to preclude a single underlying neurochemical mechanism.

1.2 Neurophysiology of Focal Seizure Generation

In 1964, Matsumoto and Ajmone Marsan described the chronology of electrophysiological changes in cells made

epileptiform by topical application of penicillin (PG) to the cortex of cats during both interictal (between seizures) and ictal (during seizures) episodes. PG application resulted in paroxysmal discharges recorded from a cortical electroencephalogram (EEG). The intracellular correlate of the cortical discharge was a large 20-50 mv depolarization lasting 50-100 msec. The intracellular depolarization correlated with the surface cortical paroxysmal discharge in 96% of cells. It was named the paroxysmal depolarization shift, and was often followed by a hyperpolarizing shift (Matsumoto and Ajmone Marsan, 1964a). The hyperpolarizing shift was found to disappear at the onset of an ictal episode and a prominent afterdepolarization developed. During the tonic phase of an ictal event, the membrane potential was decreased and rhythmical oscillations with action potentials appeared above a sustained excessively depolarized membrane potential level. The clonic phase of an ictal episode was found to correspond to a slow repolarization process. The end of an ictal event was thought to be due to inactivation rather than membrane hyperpolarization (Matsumoto and Ajmone Marsan, 1964b).

While the chronology of cellular events occuring in a epileptic focus (hereafter referred to as a focus) have been studied through intracellular recordings, there is still considerable controversy as to what initiates the paroxysmal depolarization shift and mediates the interictal to ictal transition. Two views, the epileptic neuron and the

neuronal aggregate theories, have been advanced to explain the mechanism whereby a focus develops in a group of cortical neurons. According to the epileptic neuron theory, there is an alteration of the nerve membrane which leads to new modes of spike initiation in the focus. Each neuron may be considered an "epileptic neuron" since it becomes capable of generating altered response patterns (Ayala et. al., 1973).

According to the neuronal aggregate theory, the focus is composed of "normal neurons" which fire abnormally with bursts of action potentials in response to hypersynchronous synaptic input. The excessive output of such neurons leads to abnormal input to follower neurons, resulting in a focus by maintaining neuronal synchrony (Schwartzkroin and Wyler, 1980). Ajmone Marsan (1961) in studying the electrographic aspects of epileptic neuronal aggregates in the PG foci in cats concluded that it was highly improbable that a single isolated epileptic element could develop self-sustained activity. Epileptic activity could be explained, in the case of an epileptic neuronal pool, by assigning reciprocal connections and assuming reciprocal impulses impinging among its various elements. After studying the neural behavior and triggering mechanism in the cortical PG foci of cats, Matsumoto et. al. (1969) concluded that the paroxysmal depolarization shift was a population phenomenum. Under resting conditions, when no cortical paroxysm was present, it was impossible to identify neurons of the penicillin-induced epileptic focus as potentially

epileptic. The paroxysmal depolarization shift was produced by continuous synaptic reexcitation of the neuron including distal and proximal portions of the dendrites, apparently as a result of very effective coupling among neurons in the focus.

1.3 Animal Models of Epilepsy

No experimental model developed to date is a totally adequate substitute for human epilepsy. For this reason, it is important that the scientific investigator of subhuman organisms utilize a model that is as relevant and appropriate to the study of human epilepsy as possible. Wada (1977) outlined the criteria which the ideal animal of epilepsy should meet:

1. The animal model should allow precise experimental control over both the area and size of the epileptogenic lesion to be created.
2. The introduction of destructive pathologic conditions should be avoided.
3. The animal model should allow accurate experimental control over the chronology of events leading to seizure development.
4. The seizure should be precipitated by a discrete and identifiable experimental event.

A vast variety of animal models of human epilepsy exist. These experimental models can be classified as

exibiting generalized convulsant, generalized absence, generalized myoclonic or partial (focal) seizures induced by a variety of agents. Focal seizures, have been widely studied due to both the high incidence of this type of seizure relative to other forms and the ease with which they can be produced (Prince, 1978).

The convulsant action of PG applied topically to the cerebral cortex of monkeys and cats was first reported by Walker and Johnson (1945). The ability of topically applied PG to create an epileptic focus in cortical neurons has been confirmed in the cat (Matsumoto et. al., 1969; Stark et. al., 1972; Edmonds and Stark, 1974), squirrel monkey (Matsumoto and Ajmone Marson, 1964) and the rat (Edmonds et. al., 1974). Lockton and Holmes (1980) found that the region of the cerebral cortex of urethane anaesthetized rats within the span of layers III and V (0.5-0.8 mm below the cortex) were particularly sensitive to the epileptogenic effect of PG applied electrophoretically from glass microelectrodes. Neuman (1986a) found that the pressure ejection of PG at a depth of 1.0 mm from the cortical surface (cortical layer IV-V) of urethane anaesthetized rats resulted in the appearance of focal epileptiform activity (FEA). In these experiments FEA was thought to represent a larval form of interictal spikes, rather than represent a ictal episode.

PG is considered a good choice as a means of inducing a focus (Edmonds et. al., 1974). Although cobalt is considered to be the most epileptogenic of the topically

applied heavy metals (Colasanti and Craig, 1973), heavy metals tend to disrupt cortical cytoarchitecture while cytoarchitectural disruption following the topical application of PG is not a common finding.

Kindling is an experimental model of epilepsy in which the brief application of subconvulsive nonpolarizing electrical current to discrete regions of the brain results in progressive intensification of seizure activity culminating in a generalized seizure (Racine et. al., 1979). This phenomenon was first reported by Goddard et. al. (1969) to occur in the rat, cat and monkey. In rats stimulated in the amygdala, the initial stimulus often elicits focal electrical seizure activity, but generalized seizures develop with repeated stimulation (McNamara, 1984). While kindling creates the predictable epileptogenic reorganization of brain function in time and space without causing structural damage to the cerebral tissue (Wada, 1977), it is a less practical manner of inducing focal seizures in cortical neurons of acute experimental animal preparations than the topical application of PG.

Cortical freezing has been reported to induce focal seizures in animal preparations. Variations of this procedure include the application of a metallic freezing probe to the cortex (Stalmaster and Hanna, 1972), application of dichlorodifluoromethane spray to the pial surface of gyrus posterior lateralis (visual cortex) (Hori et. al., 1979), and application of ethyl chloride spray (Lewin et. al., 1969).

In the cat, spiking activity associated with focal seizures develop in the cortex within 10 to 60 minutes of cortical freezing (Hori et. al., 1978), while the rat is relatively more resistant to cortical freezing (Dow et. al., 1962). In the rat, focal epileptic seizures usually develop within eight hours of application of ethyl chloride spray to the cortex (Lewin and McCrimmon, 1967).

There are several animal models with a genetically lowered threshold for the initiation of focal seizures including the tottering mouse (Noebels, 1979; Noebels and Sidman, 1979) and the epileptic beagle dog (Edmonds et. al., 1979). Both display a lower threshold for focal seizure initiation than their uneffected littermates. Focal seizures in these two animal models, however, can not be evoked by any form of sensory stimulation, but instead require the application of a lower than normal dose of seizure producing agent.

1.4 Neurotransmitters and Their Role in Seizures

Homeostatic abnormalities in the concentrations of many chemicals including water, electrolytes, blood gases, vitamins, and hormones are known to influence seizure susceptibility (Miller, 1981). Some of these imbalances may exert more fundamental influences on neuronal function than alterations in the synthesis, storage or release of neurotransmitters. Neurotransmitters, however, form a

better bridge between the biochemistry and physiology of the central nervous system if attention is focused on particular regions of the brain involved in particular syndromes of epilepsy (Maynert et. al., 1975).

1.41 Gamma-aminobutyric Acid (GABA)

The role of GABA in seizure disorders is not clearly understood, although there is considerable evidence that it is involved. The evidence favoring an involvement of GABA in topically induced experimental epilepsy is strong (Craig, 1984). Guerrero-Figueroa et. al. (1964) found that the topical application of GABA to the hippocampus inhibited alumina cream spontaneous epileptiform activity in primary and secondary foci. Stach and Kacz (1977) concluded that compounds that stimulate GABA receptors in ouabain-induced rabbits seem to possess antiepileptic properties. Koyama (1972) found that the concentration of GABA was reduced in cortical tissue of cats adjacent to the site of a cobalt lesion 1 day following its application. Van Gelden and Courtois (1972) found that 7 days following the application of cobalt powder to the anterior motor cortex of the cat, the adjacent epileptigenic region was deficient in GABA. A clear correlation was found to exist between the severity of epilepsy and the extent to which the concentration of GABA was reduced. In the primary focus GABA was reduced 5-10 days following the implantation of cobalt pellets in the frontal cortex of rats, whereas the GABA synthetic enzyme glutamate

decarboxylase (GAD) was significantly reduced in both the primary and secondary focus 4-8 days following implantation of cobalt (Emson and Joseph, 1975). Ribak et. al., (1979) found a highly significant numerical decrease of GAD-positive nerve terminals, indicating a functional loss of GABAergic inhibitory synapses, in monkeys made epileptic by cortical application of alumina gel. Ribak and Reiffenstein (1982) showed a similar loss of GABAergic inhibitory synapses in denervated cat cortex. Ross and Craig (1981) found that a reduction in GABA uptake occured following cobalt application and that these effects were greatest at or near the peak seizure activity. It was thought that the capacity of brain cells to take up GABA more efficiently at later time periods following cobalt administration was due to a regeneration of GABAergic nerve terminals.

1.42 Catecholamines

The inference that cerebral catecholamines (norepinephrine [NE] and dopamine [DA]) participate in seizure suppression has been reinforced by studies examining the effects of central application of 6-hydroxydopamine (6-OHDA), a drug that can produce selective damage to catecholaminergic neurons. NE and DA are thought to differentially participate in the suppression of different seizure types. The enhanced convulsive response to pentylenetetrazol observed following treatment with 6-OHDA is related to the destruction of the NE system, and not to DA,

since increasing the depletion of DA using a monoamine oxidase inhibitor (MAOI) did not greatly enhance the effect produced by 6-OHDA alone (Corcoran et. al., 1974; McIntyre et. al., 1979). In amygdala kindled rats treated with a MAOI, seizures developed significantly faster than rats treated only with 6-OHDA. This effect was thought to be a function either of a combined depletion of NE and DA or a reduction of DA alone (Corcoran et. al., 1974). Sato and Nakashima (1975) concluded that seizure susceptibilty in hippocampal and amygdaloid kindling is based on a marked depletion of NE and DA. The seizure state induced by amygdaloid kindling is accompanied by a significant depletion of NE in the hippocampus, midbrain, limbic lobes and frontal cortex while no changes in the level of NE occur in the hypothalmus, brain stem or basal ganglia (Callaghan and Schwark, 1979). Dopamine levels were not affected in any brain region. Alpha-methyl-p-tyrosine and disulfiram (drugs which deplete NE by reducing its synthesis) as well as propranolol, which impairs noradrenergic mechanisms through an action on beta-adrenergic receptors, increased the duration of afterdischarges accompanying the seizures. Drugs affecting dopaminergic mechanisms, such as pimozide and apomorphine, and drugs acting on alpha-adrenergic receptors, such as phenoxybenzamine and clonidine, had no effect on the duration of afterdischarges accompanying seizures. Mohr and Corcoran (1981) concluded that the facilitation of amygaloid kindling produced by depletion of

NE by 6-OHDA is due to a disinhibition of the spread of epileptiform activity from the stimulated amygdala. NE depletion was found not to effect either the threshold for after discharge or the duration of the first afterdischarge at the primary focus. Engel and Sharpless (1977) found that amygdaloid kindling produced a definite, specific, long-lasting (measured one month after the last kindled convulsion) decrease in local dopamine concentration. This could be explained either by a persistent increase in dopamine release and metabolism or by a persistent decrease in dopamine production. A decrease in NE which occured was thought to not be greater than that which would be expected to occur following electrode placement in the amygdala.

Quattrone et. al., (1978) determined that NE was involved in the control of electroshock seizure susceptibility in rats since no change in electroconvulsion threshold were found in animals in which NE but not DA neurons were protected by the selective NE reuptake inhibitor desmethylimipramine (DMI) pretreatment from the action of 6-OHDA. It was further suggested that the anticonvulsant action of carbamazepine, diphenylhydantoin and phenobarbital is related to NE since protecting NE neurons with DMI led to no significant difference between 6-OHDA treated animals and their controls.

Colasanti and Craig (1973) reported no change in NE or DA levels or turnover in cobalt-induced epileptic rats. A possible flaw in this experiment, however, is that whole

brain rather than specific brain regions were analyzed for catecholamine content. Trottier et. al., (1981) studied alterations in the cortical NE system following the application of cobalt to the sensorimotor cortex of the rat using the glyoxylic histochemical fluorescence method combined with biochemical analysis of cortical NE content. A decrease in the density of NE terminals in the perifocal area was found before the onset of spike activity. NE levels were lowest when the first epileptic discharges were recorded. At the end of the epileptic syndrome, sprouting of noradrenergic fibres in the perifocal area coincided with an increase in the NE levels.

Neuman (1986b) found that electrical stimulation of the locus ceruleus, a major NE containing nucleus in the brainstem, or of its axons projecting to the forebrain, the dorsal noradrenergic bundle, suppressed PG-induced FEA. This suggested NE involvement in the suppression of interictal spiking.

1.43 Serotonin

The possible importance of 5-hydroxytryptamine (serotonin, 5-HT) as a modulator of seizure susceptibility has been repeatedly suggested in the past 30 years. Unfortunately some of the experimental evidence that appears to include or exclude a role for serotonin in various seizure states is clouded by the use of nonspecific drugs and procedures (lesion studies). Drugs that produce a marked

reduction in brain 5-HT content can be grouped into three categories in relation to their mechanisms of action: 1) drugs that release the stores of 5-HT, e.g. reserpine and p-chloroamphetamine (PCA); 2) drugs that inhibit the synthesis of 5-HT, e.g. p-chlorophenylalanine (PCPA); and 3) drugs that have a cytotoxic effect on 5-HT neurons, e.g. 5,7-dihydroxytryptamine (5,7-DHT) (Sanders-Bush and Massari, 1977).

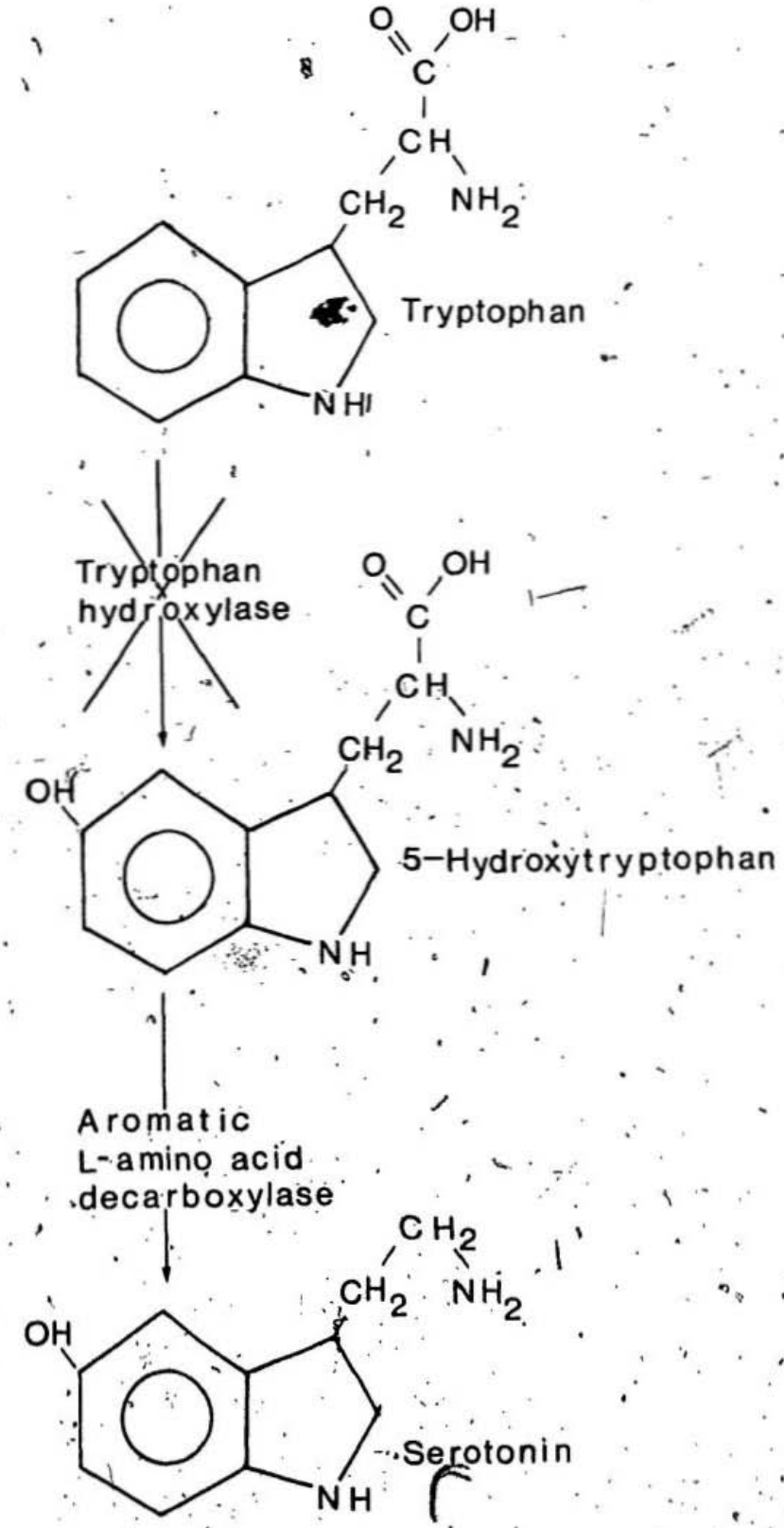
The possible importance of 5-HT as a modulator of seizure susceptibility was suggested in 1954 by Chen et. al. who observed seizure facilitatory effects by reserpine in mice subjected to electroshock and pentylenetetrazol induced convulsions. Reserpine used as a drug to deplete 5-HT has serious limitations, the most important being its nonspecific effects. Azzaro et. al. (1972) concluded that both 5-HT and catecholamines were involved in the reserpine-induced reduction in threshold for minimal electroshock seizures in the mouse. Not only is 5-HT in the brain depleted to negligible levels but also the levels of NE and DA are reduced in association with the concomitant increase in 5-hydroxyindoleacetic acid (5-HIAA), the principle metabolite of 5-HT and the deaminated metabolites of NE and DA (Sanders-Bush and Massari, 1977).

The administration of PCA causes a marked reduction of both 5-HT, 5-HIAA and tryptophan hydroxylase (Sanders-Bush et. al., 1975) without altering the levels of NE and DA (Pletscher et. al., 1964). Pharmacological tests to

determine the role of 5-HT in seizure production through the use of PCA in amygdala kindled rats found that PCA did not reduce seizure threshold. PCA was thought to reduce the inhibiting effect of 5-HT on the seizure mechanism, but this seizure mechanism could not be sensitized any further than had already been accomplished through kindling (Siegel and Murphy, 1979).

The hydroxylation of tryptophan by tryptophan hydroxylase is the rate limiting step in the synthesis of 5-HT, and drugs such as PCPA that reduce the synthesis of 5-HT likely do so by modifying the activity of this enzyme (Fig 1) directly or indirectly by decreasing the availability of the substrate tryptophan (Sanders-Bush and Massari, 1977). Koe and Weissman (1966) concluded that PCPA was a potent and selective depletor of brain 5-HT and 5-HIAA in mice, rats and dogs. PCPA, however, is not an entirely selective depletor of 5-HT, since NE levels are slightly but consistently depleted in PCPA treated animals (Welch and Welch, 1967; Kilian and Frey, 1973). De La Torre and Mullan (1970) found that treatment with rats with PCPA enchanced the susceptibility to pentylenetetrazol induced seizures while treatment with the 5-HT precursor 5-hydroxytryptophan (5-HTP) decreased the susceptibility to pentylenetetrazol induced seizures. PCPA was found by Buterbaugh (1977) to increase the susceptibility of rats to maximal electroshock. The activation by seizure discharge of one or more central serotonergic systems was thought to be an important

Figure 1. The mechanism of depletion of 5-HT by PCPA. PCPA is thought to deplete 5-HT by inhibiting the hydroxylation of the enzyme tryptophan hydroxylase.


O
OH
C
CH
CH2
NH2
Tryptophan
NH
Tryptophan
hydroxylase
OH
5–Hydroxytryptophan
Aromatic
L-amino acid
decarboxylase
Serotonin

functional determinant of the convulsive response pattern to maximal electroshock stimulation.

5,7-DHT is a powerful neurotoxin that destroys monoamine-containing cells including 5-HT in the CNS. The usefulness of 5,7-DHT has been somewhat limited due to its non-selective toxicity for both indoleamines and catecholamine neurons. The administration of a specific NE reuptake blocker prior to intracisternally administering 5,7-DHT appears to provide increased selectivity to lesion 5-HT neurons and spare catecholamine neurons (Stewart et. al., 1976). Browning et. al., (1978) found a seizure-facilitating effect of 5,7-DHT administered after the NE reuptake blocker protriptyline, in pentylenetetrazol and electrically induced seizures in rats. Electrical lesions of the dorsal and median raphe nuclei, which produced an 80% deficit in forebrain 5-HT, failed to alter the response of the animals to chemically and electrically induced seizures. It was thought that either a specific group of 5-HT neurons, not among the neurons damaged by lesioning the dorsal and median raphe nuclei, must be responsible for antagonizing seizures or that 5,7-DHT may have seizure facilitating effects unrelated to its action on 5-HT neurons. Crunelli et. al., (1979) found that the intraventricular administration of 5,7-DHT following an intraperitoneal (i.p.) injection of DMI did not change seizure susceptibility to electrically induced convulsions 10 or 30 days after surgery dispite marked selective depletion of 5-HT in the brain and spinal

cord. Electrolytic and chemical (5,7-DHT) lesions of the dorsal raphe also did not alter the seizure susceptibility.

While lesions of the raphe nuclei failed to influence seizure susceptibility, Kovacs and Zoll (1974) found that electrical stimulation of the median raphe caused the inhibition of both amygdala kindled and pentylenetetrazol induced seizures in rats. Since stimulation of the median raphe was known to release 5-HT (Aghajanian et. al., 1967) this suggested that 5-HT was capable of inhibiting seizures.

1.5 The Effect of Noxious Stimulation on Epileptiform Activity

The ability of noxious stimulation (NS) to suppress or lessen the severity of epileptic seizures has been known for over 1700 years. In the 2nd century A.D., Greek physicians (most notably Galen and Aretaeus) found that the application of ligatures to limbs of patients whom had experienced an "aura" lessened the severity and postponed the onset of seizures (Temkin, 1971).

The ability of NS to suppress spike and wave activity is thought to be due to its ability to increase cortical arousal. Epileptiform activity in humans and laboratory animals is subject to suppression by numerous procedures which increase cortical arousal, including NS (Pollen et. al., 1963; Myslobodsky, 1976; Gloor, 1984). The application of NS readily produces a suppression of epileptiform activity

in pentobarbital anaesthetized cats (Pollen et. al., 1963) and urethane anaesthetized rats (Neuman, 1986a). NS is known to activate cholinergic (Dutar et. al., 1985), serotonergic (Segal, 1979), dopaminergic (Barasi, 1979) and noradrenergic (Korf et. al., 1974) systems in the CNS all of which have been postulated to play a role in modulating seizure activity.

The suppression of focal epileptiform activity (FEA) by NS has been suggested to be mediated, at least in part, by 5-HT. Neuman (1986a) found that NS was ineffective in suppressing PG-induced FEA in both reserpine and PCPA pretreated rats. However, the i.p. administration of L-5-hydroxytryptophan (5-HTP), a 5-HT precursor, was found to restore the suppression of FEA by NS. It was therefore concluded that the suppression of FEA by NS was mediated, at least in part, by 5-HT. Clearly the case for 5-HT involvement in the suppression of FEA by NS would be strengthened if the 5-HT receptor subtype involved and the site of action of this effect could be determined.

1.6 Serotonergic Raphe-Cortical Projections in the Rat

The raphe nuclei are a collection of predominantly midline neurons within the brainstem that contain the largest concentration of serotonergic perikarya in the brain and are the primary source of serotonergic innervation of much of the neuraxis (O'Hearn and Molliver, 1984). It has been

suggested that the raphe nuclei play an important role in mediating the effects of 5-HT on sleep, neuroendocrine regulation, sexuality, aggression, seizure threshold and pain responses (Conrad et. al., 1974).

Ascending projections to the cerebral cortex are prominent among the projections of the raphe nuclei. Fluorescent histochemistry was used by Fuxe (1965) and Ungerstedt (1971) to demonstrate that the dorsal raphe and the median raphe are the primary sources of cerebral 5-HT. Direct dorsal raphe and median raphe neocortical projections have been confirmed using horseradish peroxidase (Bentivoglio et. al., 1978; Tohyama et. al. 1980), autoradiographic (Conrad et. al., 1974; Azmitia and Segal, 1978) and fluorescent retrograde dye tracing techniques (Van der Kooy and Hattori, 1980).

Using the fluorescent retrograde dye tracing technique, O'Hearn and Molliver (1984) determined that the dorsal raphe nucleus innervated all regions of the cerebral cortex. Subsets of raphe cells were postulated to act differentially upon particular cortical areas, despite the observation that the dorsal raphe appears to exert its greatest effect quantitatively upon the frontal cortex. The differential projections are thought to provide the dorsal raphe with the capacity to have selective actions upon individual functional areas of the neocortex with a particularly strong effect on frontal lobe function. The median raphe, on the other hand, appears to have widespread

and bilateral projections involving perhaps a more generalized effect on cortical structures.

Techniques employing either the retrograde transport of the enzyme horseradish peroxidase (Watkins et. al., 1980) or the anterograde transport of tritiated amino acids (Goode et. al., 1980) have demonstrated that raphe pallidus, raphe magnus and raphe obscurus neurons project primarily to the spinal cord via the dorsolateral funiculus, while the more caudal cell groups project primarily to the spinal cord via the ventral funiculus.

1.7 Serotonin Receptors in the Rat CNS

Early studies using 5-HT focused primarily on its action on isolated peripheral tissues. 5-HT was first discovered as an endogenous biogenic amine present in enterochromaffin cells in the gut. The first classification of multiple 5-HT receptors was by Gaddum and Picarelli (1957) for the guinea pig ileum. 5-HT receptors were classified as D or M on the bases of their antagonism by phenoxybenzamine (Dibenzyline) or morphine. The D receptor was blocked by d-lysergic acid diethylamide (LSD) and its analogues while the M receptor was blocked by atropine as well as morphine.

1.71 Electrophysiologically Characterized 5-HT Receptors

Aghajanian (1981) proposed the existence of three distinct 5-HT receptors based on neuronal responses to 5-HT

as determined by unit recording.

S1 Receptor

The S1 receptor is a central 5-HT receptor whose properties resemble 5-HT receptors in the periphery. The activation of this receptor by 5-HT or 5-HT agonists facilitates the depolarizing action of excitatory amino acids in both facial motorneurons (McCall and Aghajanian, 1979) and spinal motorneurons (White and Neuman, 1980). The physiological action of 5-HT mediated by these receptors is that of a modulator nature in which the electrical excitability of postsynaptic neurons is increased. Classical 5-HT antagonists such as methysergide, cinanserin and cyproheptadine which are effective against 5-HT receptors, also block the S1 receptor (Aghajanian, 1981).

S2 Receptor

The S2 receptor is a presynaptic autoreceptor, i.e. a receptor mediating the response of a neuron to its own neurotransmitter (Wang and Aghajanian, 1978). These receptors are thought to mediate collateral inhibition within the rostral raphe nuclei either through a direct inhibitory mechanism or by modulating pacemaker activity. Classical 5-HT antagonists are without effect on S2 receptors while LSD and other indoleamine hallucinogens are powerful agonists at this receptor (Aghajanian, 1981).

S3 Receptor

The postsynaptic S3 receptor is located in many parts of the forebrain including the limbic system and secondary visual

areas. The S3 receptor suppresses rather than facilates neuronal activity. LSD is a weak partial agonist at S3 receptors and classical 5-HT antagonists are not consistently effective in blocking these responses (Aghajanian, 1981).

1.72 Classification of 5-HT Binding Sites by the Radioligand Method

Radioligand studies provide an accurate measure of binding site density and drug affinity for specific membrane recognition sites (Snyder, 1983). The most widely used classification of 5-HT binding sites in the CNS was initially proposed by Peroutka and Snyder (1979). They observed that [^{3}H] 5-HT and [^{3}H] spiroperidol label distinct populations of 5-HT binding sites in the rat brain, which they termed 5-HT$_1$ and 5-HT$_2$ respectively, whereas [^{3}H] LSD appear to bind to both binding sites to a similar extent.

The 5-HT$_1$ sites can be further divided into subgroups, 5-HT$_{1A}$ and 5-HT$_{1B}$ (Pedigo et. al., 1981). The definition is based on their affinities for the neuroleptic spiperone, 5-HT$_{1A}$ sites have a high affinity for spiperone (K_i=2-3 nm) whereas 5-HT$_{1B}$ sites have a low affinity for spiperone (K_i=35 um). Using autoradiography, Deshmukh et. al., (1983) demonstrated both the occurence of and regional differences in the pharmacological sensitivity of 5-HT$_{1A}$ and 5-HT$_{1B}$ binding sites. Areas such as the hippocampus, septum and cerebral cortex appear to be more sensitive to inhibition of [^{3}H] 5-HT binding by spiperone than other areas suggesting

that these regions have significant proportions of 5-HT_{1A} sites. Other areas such as the caudate nucleus, superior colliculus, lateral geniculate body and substantia nigra appear resistant to inhibition of binding by spiperone, suggesting that they contain the 5-HT_{1B} type of binding site. A further subtype of 5-HT_1 binding sites, designated 5-HT_{1C}, is concentrated in the choroid plexis of the pig (Pazos et. al., 1985) and rat (Pazos and Palacios, 1985). It is now possible to identify four distinct 5-HT_1 binding sites with specific radioligands. 5-HT_{1A} sites are labelled by [^{3}H] 8-hydroxy-2-(di-n-propylamino) tetralin ([^{3}H] 8-OH-DPAT) (Pazos et. al., 1985), 5-HT_{1B} sites are labelled by [^{125}I] cyanopindolol (in the presence of 30 uM isoprenaline in order to exclude beta receptor binding) (Engel et. al., 1986) and 5-HT_{1C} receptors are labelled by [^{3}H] mesulergine (Pazos and Palacios, 1985). Recently, Heuring and Peroutka (1987) have found that [^{3}H] 5-HT labels a population of binding sites, designated 5-HT_{1D}, in bovine caudate that is distinct from previously defined 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} binding sites.

In addition to [^{3}H] spiperone and [^{3}H] LSD, described as 5-HT_2 ligands by Peroutka and Snyder (1979), other [^{3}H]-labelled compounds have been developed in the search for more specific ligands for 5-HT_2 binding sites. [^{3}H] mianserin (Peroutka and Snyder, 1981), [^{3}H] ketanserin (Leyson et. al., 1981) and [^{3}H] ritanserin (Leysen et. al., 1985) have recently been assayed. [^{3}H] mianserin labels 5-HT_2 binding sites and histamine-H_1 binding sites concomitantly while

[^{3}H] ketanserin and [^{3}H] ritanserin have high affinity for 5-HT_2 binding sites. The affinities of such 5-HT_2 antagonists correlate well with those at "D" receptors.

The "M" receptors originally described by Gaddum and Picarelli (1957) do not fall into either the 5-HT_1 or 5-HT_2 category. Fozard et. al. (1979) found that "M" receptors similar to those in the guinea-pig ileum, mediate the release of NE from sympathetic nerves innervating the rabbit heart. Cocaine is a weak but nevertheless specific antagonist at these receptors (Fozard et. al., 1979). Such studies led to the development of potent, selective 5-HT_3 ("M") antagonists such as MDL 72 222 (Fozard, 1984) and ICS 205-930 (Richardson et. al., 1985). 5-HT_3 binding sites have not been localized in the CNS but are located on peripheral neurons where they are thought to mediate the depolarizing action of 5-HT.

1.73 Radioligand Versus Physiological Studies

Before a binding site can be considered to be a receptor, the correlation of radioligand data with physiological response is necessary. Numerous problems exist when such correlations are attempted (Peroutka, 1984):

1. Only a few pharmacological agents are normally analyzed in physiological studies. Correlations are only valid when they include a large series of drugs, preferably comprising members of different chemical classes, with activities distributed over a wide potency range (Leysen, 1984).

2. Pharmacological agents may be hindered from reaching the receptor site in tissue preparations, such as cortical

slices. In in vivo studies the possible problems of distribution, absorption and metabolism are even more important.

3. Large concentrations of exogenous neurotransmitters may act simultaneously at multiple sites. Therefore, attributing the observed effect of an agonist to a single receptor site may be erroneous.

1.74 The Degree of Correlation Between Radioligand and Physiological Studies

At present there is no satisfactory congruence between the physiological characterization of postsynaptic 5-HT receptors and the identification, by the radioligand method, of 5-HT binding sites. Since classical antagonists have a high affinity for both 5-HT_1 and 5-HT_2 binding sites but fail to antagonize S_3-mediated responses, the S_3 receptor would appear to be distinct from these two sites (De Montigny et. al., 1984). Methiothepin has a high affinity for both 5-HT_1 and 5-HT_2 binding sites (Richardson and Engel, 1986) but fails to block the S_1 mediated responses in the facial motor nucleus (McCall and Aghajanian, 1980) suggesting that neither binding site corresponds to the S_1 receptor (De Montigny et. al., 1984).

At present a role for 5-HT_1 binding sites has not been demonstrated and with existing information they cannot be considered as receptor sites (Leysen, 1984). Some behavioral and physiological actions of 5-HT_2 binding sites, however, have been determined. In rats, administration of

5-HT precursors or 5-HT mimetic agents elicits head twitches, forepaw treading, myoclonus and impairment of blood circulation (Leysen et. al., 1984). Studies utilizing a large series of drugs demonstrated highly significant correlations between the potencies of drugs to antagonize behavioral excitation induced by serotonin and binding affinities for 5-HT_2 binding sites (Leysen, 1984).

A much better correlation exists between electrophysiological characterization of S_2 receptors and the radioligand characterized 5-HT binding sites. In both paradigms LSD acts as an agonist (Haigler and Aghajanian, 1974). Functionally LSD decreases 5-HT release by acting at the terminal autoreceptor designated as corresponding to a 5-HT_{1B} binding site (Engel et. al., 1986). Methiothepin, on the other hand, acts as an antagonist at the terminal autoreceptor (Pettibone and Pflueger, 1984; Chaput et. al., 1986).

1.8 Rationale for the Present Study

The results of Neuman (1986a) implicate 5-HT involvement in mediating the suppression of FEA by NS. One way to strengthen the role for 5-HT in this process is to localize the site of action of this effect and identify the 5-HT receptor type involved.

The main working hypothesis of this thesis is that an increased release of 5-HT at the cortical level mediates the

suppression of FEA by NS. Since cortically projecting raphe neurons arise mainly from the dorsal and median raphe nuclei, it is likely that one or both of these structures is involved in mediating these effects. This thesis summarizes several types of experiments that were done to test this hypothesis:

1. Experiments aimed at simulating the effects of NS by directly increasing cortical concentrations of 5-HT or, conversely, blocking the suppression of FEA by NS by reducing the cortical release of 5-HT.
2. Experiments aimed at antagonizing the effect of NS on FEA by decreasing raphe firing by the local pressure injection of 5-HT agonists.
3. Experiments aimed at determining the 5-HT receptor type involved in mediating the suppression of FEA by NS by the i.v. injection of 5-HT agonists and antagonists.

Chapter 2

Materials and Methods

2.1 Animals

Male and female Sprague-Dawley rats were obtained from Canadian Hybrid Farms in Halls Harbour, Nova Scotia. The rats were communally housed in cages at the animal care facility of the Health Sciences Centre and kept on a 12 hr. light/12 hr. dark cycle. Food (Purina Rat Chow) and water were provided ad libitum.

2.2 Surgery

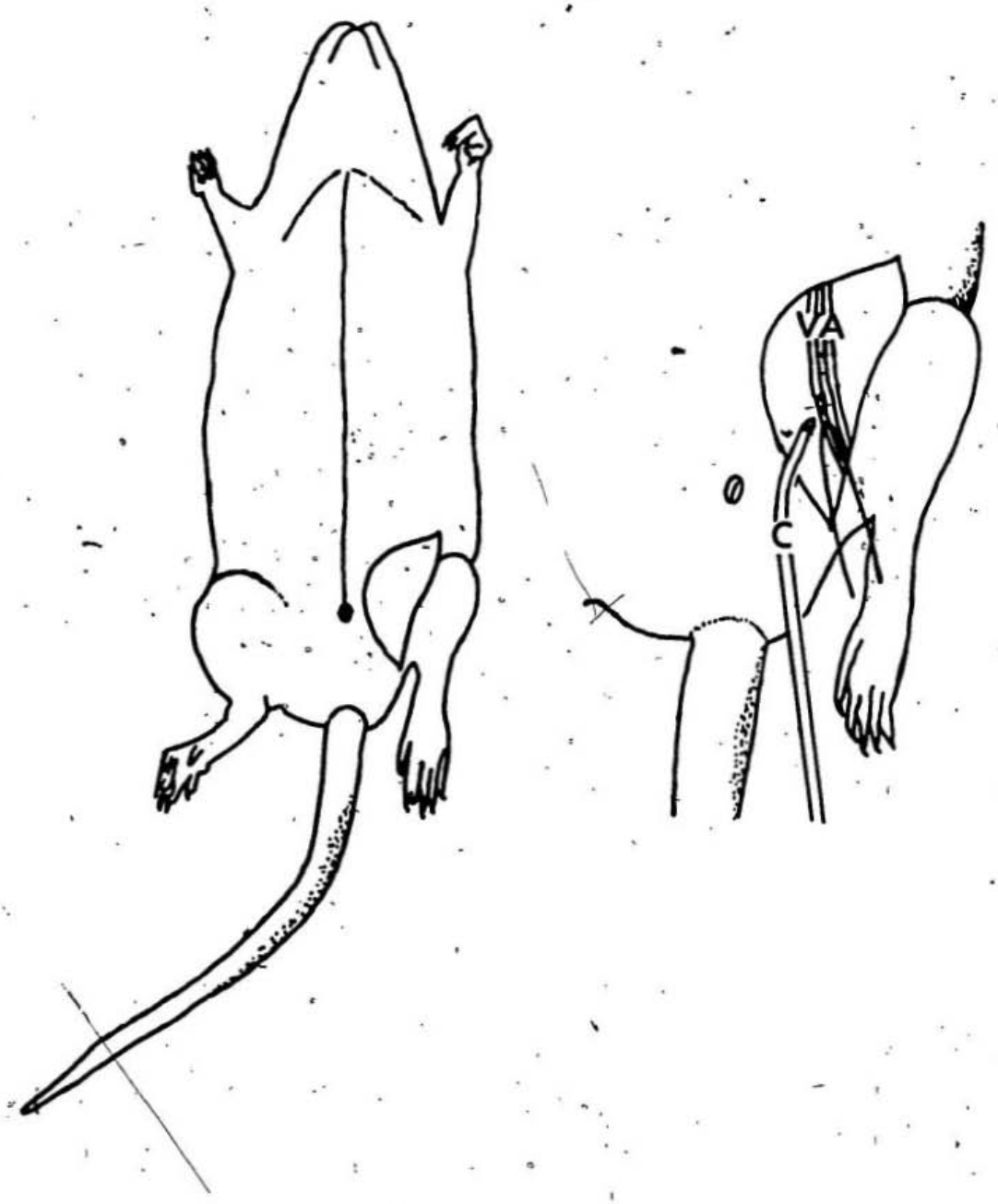
2.21 Preparatory Surgery

Rats between 200g-420g at the time of experimentation were weighed and anaesthetized by intraperitoneal (i.p.) injection of urethane (1.25 g/kg). For experiments requiring the intra-venous (i.v.) injection of drugs, the femoral vein of the left hind leg was cannulated with PE-50 tubing (Fig 2).

2.22 General Surgery

Rats were mounted in a stereotaxic frame and an incision was made along the midline of the skull to expose lamba and bregma. An area of the cortex was exposed (2-5 mm

Figure 2. The cannulation of the femoral vein of the rat with PE-50 tubing. The cannula (C) is shown about to be inserted into the left femoral vein (V) adjacent to the left femoral artery (A).


VA
C

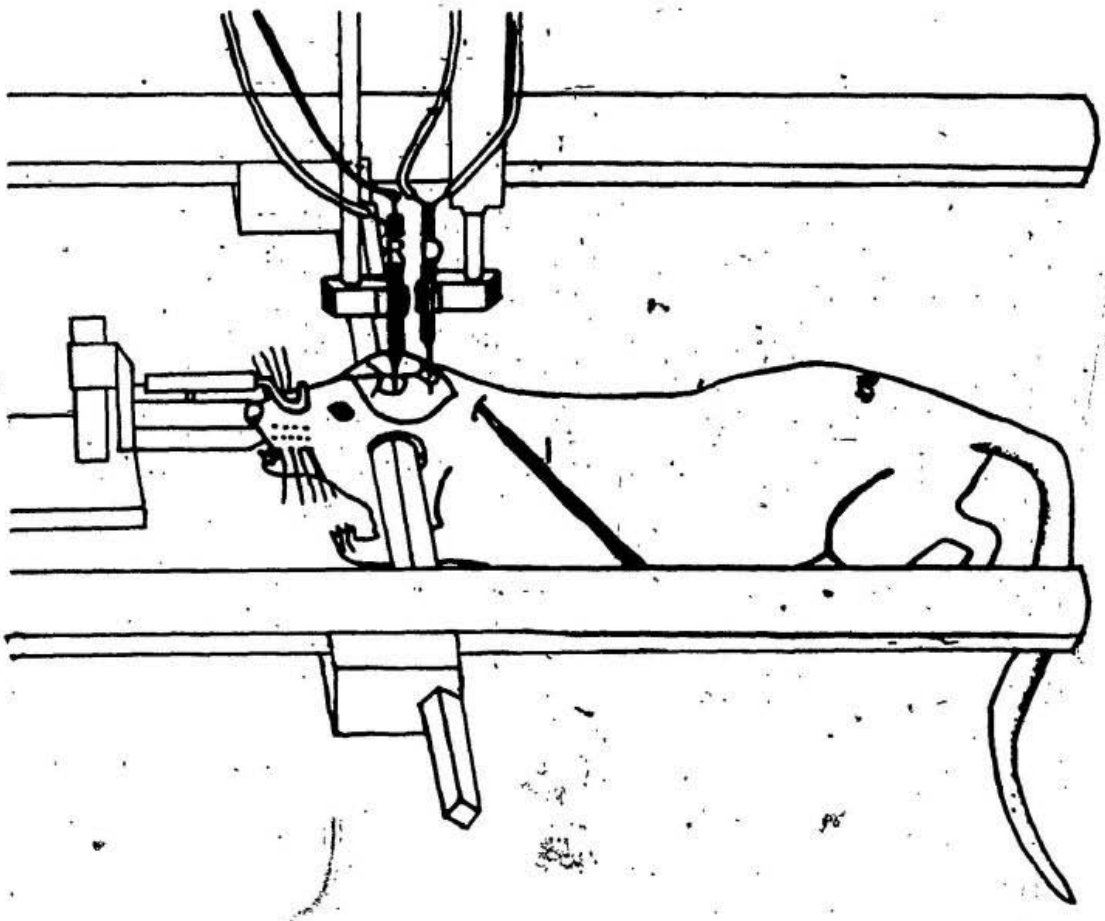
caudal to bregma and 1-3 mm lateral) using a dental drill and the dura reflected to allow placement of an electrode for recording and drug application. In animals in which electrodes were to be placed in the dorsal raphe for drug application a second burr hole was drilled exposing an area on the midline 0.5 mm anterior to lambda. An Ag/AgCl reference electrode was placed under the skin of the neck (Fig 3). Pressure points and wound margins were coated with 2% xylocaine jelly or infiltrated with a 2% solution of lidocaine. After electrode placement the brain was covered with warm agar (2%) in saline. Core temperature was monitored and maintained between 36 and 37.6 C by a servo-controlled heating system.

2.3 Micropipettes

2.31 Intracortical Recording and Drug Delivery

Intracortical recording and drug delivery were made using 5 barrel glass micropipettes (Omega Dot, Glass Company of America) pulled on a Narishige microelectrode puller and broken back to 15 µm tips. The centre barrel contained 4 M NaCl and was used for recording. A second barrel was filled with 100 mM PG for application by pressure ejection. PG was dissolved in Ringer's solution (pH=7.4) in which the isotonicity was maintained by deleting 100 mM NaCl from the Ringer's. The remaining 3 barrels were available for the pressure ejection of drugs or were filled with saline. The

Figure 3. The stereotaxic setup of a rat during the intracortical recording procedure. Shown are the 5 barrel recording electrode (R), the 5 barrel electrode for pressure ejection of drugs (D) into the dorsal raphe region and the Ag/AgCl indifferent electrode (I) placed under the skin of the rat's neck.



micropipettes were prepared for pressure ejection using the method of Neuman (1986c). PG was ejected (35-137 kPa) using a compressed air source. Other drugs were applied by pressure pulses (50-1000 msec at 275 or 550 kPa) using a Picospritzer connected to a nitrogen gas source.

2.32 Drug Delivery to the Raphe Nuclei

5 barrel micropipettes broken back to a 15 µm tip, identical to those used for intracortical recording and drug delivery, were used to pressure eject drugs into the dorsal raphe nucleus.

2.4 Electrical Circuitry

Intracortical activity was recorded with a high impedance headstage (WPI 701), amplified, filtered between 0.1 and 75 Hz, and displayed on a Grass chart recorder. To quantify changes in the recorded activity, the signal was digitized at 303 samples/sec by a laboratory computer and the absolute value was integrated. As the signal was integrated the value of the integration was converted to an analogue signal which was displayed on another channel of the Grass recorder. The integration was reset every 2, 2.5 or 5 seconds.

2.5 Experimental Protocol

The recording electrode was placed 1 mm below the surface of the cortex and the skull surface covered with agar. Following a 30-45 minute recovery interval, the EEG recording procedure was initiated. To qualify as a suppression of FEA resulting from the application of NS and not a spontaneous event due a fluctuating level of anaesthetic (Angel, 1976) the suppression of FEA had to be concomitant with the delivery of NS and had to be repeatable. Noxious stimuli in different experiments consisted of: 1) a blunt probe applied to the tail by a regulated pressure source, solenoid valve, and automatic timer (TP); 2) square wave electrical pulses (10 Hz, 0.2-0.5 msec, 2-100 V) applied to the tail via subcutaneous needle electrodes (ETS); 3) a mini-gator clip applied to the tail (TC).

2.51 Pretreatment With PCPA

Rats requiring pretreatment with PCPA were given i.p. injections of 1.5 mmol/kg (suspended in 5% acacia) the first day and 0.5 mmol/kg each succeeding day of treatment. The duration of treatment was from one to three days with animals being tested at least 24 hours following their last injection.

2.52 I.V. Injections of Drugs

After obtaining several control responses to NS, serotonergic agonists, antagonists, or other drugs were administered i.v. and their effect on the suppression of FEA

by NS, as well as the duration of their effect noted. If a given dose of drug failed to elicit an effect, higher doses were administered. Following the completion of the experiment, 3M KCl was infused i.v. to confirm that the cannula had been in place in the femoral vein during the entire experiment. When the cannula was in place, 3M KCl rapidly killed the animal.

2.53 Intracortical Pressure Ejection of Drugs

After several control responses to NS were obtained, 5-HT, PCA or fenfluramine were pressure ejected into the cortex. The resultant effect of these drugs upon the amplitude of the FEA was noted as was any change in the duration of the suppression of FEA upon delivery of NS.

2.54 Local Pressure Ejection of Drugs Into the Raphe Nuclei

After obtaining several control responses to NS, discrete pulses of 5-HT or an appropriate 5-HT agonist were pressure ejected into the dorsal raphe nuclei. If no effect occured, higher doses (longer pulses) of drug were delivered. If an effect (blockade of the suppression of FEA by NS) occured following the pressure ejection of 5-HT or a 5-HT agonist, the duration of time between the pressure ejection of drug and effect was noted as was the duration of effect. Following recovery, the electrode was moved to a greater depth to determine if an effect could be obtained at the new site and, if so, if more or less drug was required to obtain

the effect.

2.6 Statistics

In each animal the mean (± standard error) of 20 values corresponding to FEA amplitude were integrated for periods before and during drug application. The paired Student's t test was used to compare the means for significance ($P<0.05$ was considered significant).

2.7 Immunohistochemistry

PCPA treated animals were perfused through the abdominal aorta with chilled saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and stored in fixative for 24 hours prior to being serially sectioned on a Vibratome (40 µm) in PBS and placed in PBS containing wells. Sections were processed according to the peroxidase-antiperoxidase (PAP) method of immunohistochemistry (Sternberger, 1979) with modifications for free floating sections. Sections were preincubated at room temperature for 45 minutes in 10% normal goat serum (NGS) in PBS containing 0.2% Triton X-100. The NGS was added to block any non-specific binding sites on the brain tissue to which the linking antibody (goat anti-rabbit) might attach. With the non-specific binding sites blocked each linking antibody molecule would be expected to bind to the appropriate binding

site on each primary 5-HT antibody molecule. The Triton X-100 was added to make intracellular binding sites accessible to the primary 5-HT antisera. 200 µl of the blocking solution was placed in each section containing well and the well trays placed on a aliquot mixer during the incubation period. Sections were transfered to clean well trays, with each section being placed in 200 µl of a 1:2000 dilution of primary 5-HT antisera of rabbit origin (ImmunoNuclear). Sections were incubated in the primary 5-HT antisera for 48 hours at 4 C. After 4 washes in PBS, each of 30 minutes duration, each section was placed in 200 µl of a 1:150 dilution of goat-antirabbit-antiserum (Boehringer Mannhein Biochemicals) at room temperature for 3 hours while agitated on a aliquot mixer. Sections were once again given 4 rinses of 30 minutes duration in PBS and then transfered to clean well trays, each well containing 200 µl of a 1:300 dilution of rabbit peroxidase-antiperoxidase (Sternberger Meyer Immunocytochemicals). Well trays were placed on a aliquot mixer for 2 hours at room temperature followed by overnight incubation at 4 C.

Following a final 4 washes in PBS, each of 30 minutes duration, sections were transfered to clean well trays containing 3,3'-diaminobenzadine HCl (Sigma) (0.5 mg/ml), glucose-oxidase (3.8 mg/ml; Apergillus niger Type v, Sigma) and D-glucose (Sigma) (2 mg/ml in 0.1 M phosphate buffer, pH 7.2) for a 10-15 minute duration. The 3,3'diaminobenzadine HCl served as an electron donor and hydrogen peroxide,

liberated upon the reaction of the enzyme glucose-oxidase with D-glucose, served as a substrate in the formation of insoluble brown reaction product (Clark, Downs and Primus, 1982). Hydrogen peroxide was not added directly to the 3,3'-diaminobenzadine HCl due to the amount of time (5-10 minutes) required to transfer sections from the PBS to the developing solution. The sections were mounted in PBS, air-dried, rinsed briefly in distilled water, dehydrated in ethanol, cleared in xylene and coverslipped in Eukitt.

2.8 Drugs

The following compounds were used in the various experiments comprising this thesis: baclofen (Ciba-Geigy); DL-p-chloroamphetamine (PCA, Sigma); p-chlorophenylalanine (PCPA, Sigma); desimipramine (DMI, Ciba-Geigy); fenfluramine (Fen, A-H Robins); fluoxetine (Flu, Lily); 5-hydroxytryptamine (bimaleate salt)(5-HT, Sigma); L-5-hydroxytryptophan (5-HTP, Sigma); ICS 205 930 (Sandoz); imipramine (IMI, Sigma); ketanserin (Janssen); (+)-8-hydroxy-2-(di-n-proplyamino) tetraline HBr (8-OH-DPAT, Research Biochemicals Inc.); LY 538 57 (Lily); mesulergine (Sandoz); metergoline (Farmitalia); 2-methoxyphenyl-piperazine (2-MPP, Research Biochemicals Inc.); methysergide (Sandoz); mianserin (Janssen); pizotifen (Sandoz); quipazine (Miles Laboratories, Inc.); ritanserin (Janssen); Sodium Penicillin G (PG, Sigma); spiperone

(Janssen); trifluoromethoxyphenylpiperazine (TFMPP, Research Biochemicals Inc.).

Chapter 3

Results

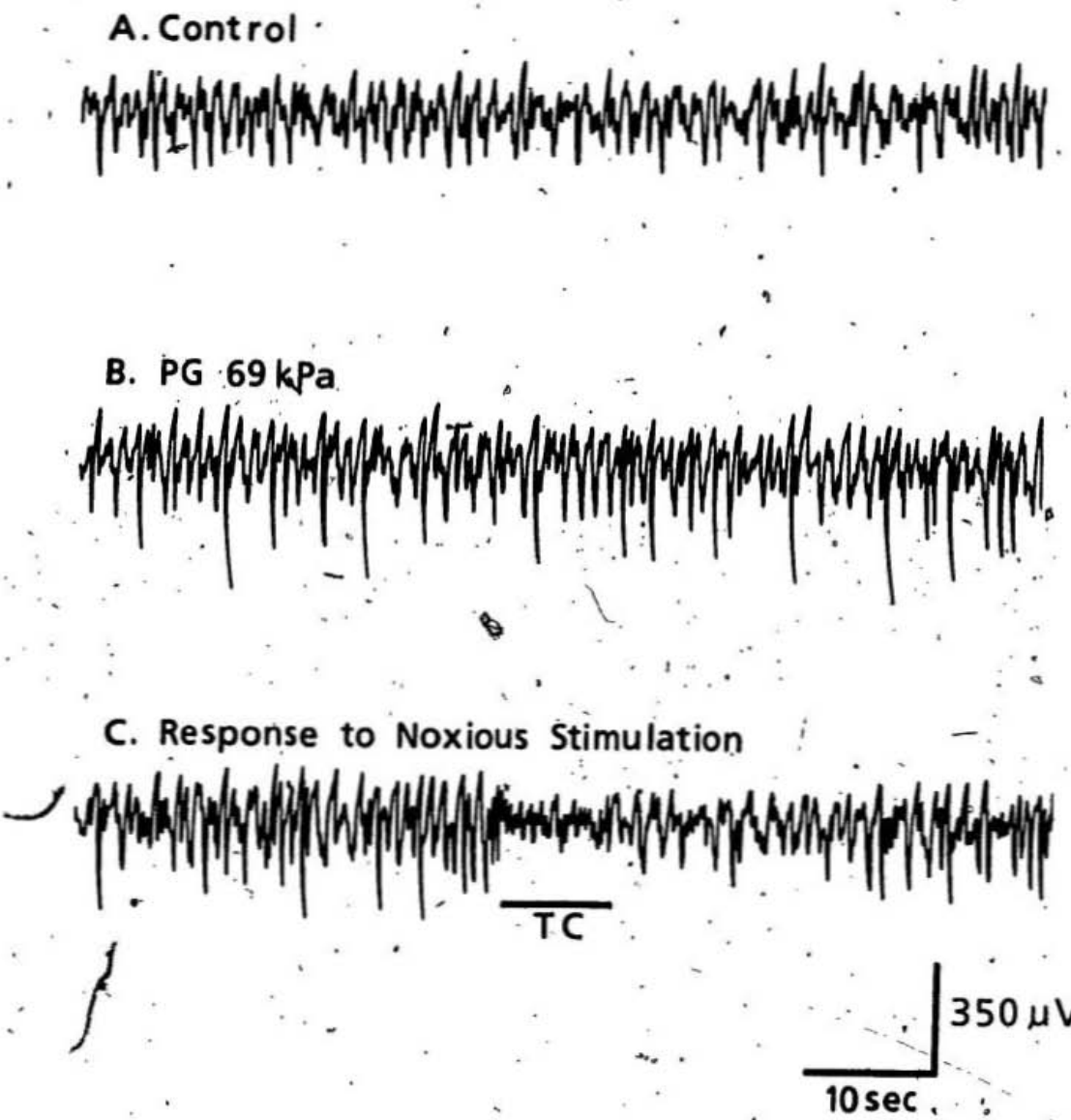
3.1 Noxious Stimulation and Suppression of FEA

Intracortical EEG activity recorded from urethane anaesthetized rats consisted of slow waves with occasional periods of desynchronization. Following the pressure ejection of PG (69-140 kPa) for a period of 1-20 min, FEA developed. It appeared to be characterized by the transformation of slow waves to sharp waves and spikes. In accord with previous observations (Neuman, 1986b), NS was found to rapidly suppress FEA, with recovery taking place following the removal of NS (Fig 4).

3.2 5-HT Agonists and the Suppression of FEA by NS

5-HT neurons contain two types of autoreceptors. One is labelled by [^{3}H] 8-OH-DPAT (Hamon et. al., 1986) and is located on the soma of 5-HT neurons in the dorsal and median raphe nuclei (Gozlan et. al., 1983; Verge et. al., 1985; Dourish et. al., 1986). Activation of this receptor by 5-HT_{1A} agonists, such as 8-OH-DPAT, inhibits spontaneous cell firing of 5-HT neurons (De Montigny et. al., 1984; Sprouse and Aghajanian, 1986). The second autoreceptor is located on terminals of 5-HT neurons projecting to the forebrain (Middlemiss, 1984; Engel et. al., 1986) and is labelled by

Figure 4. The effect of PG pressure ejected into the rat's cortex. Control (A) shows intracortical recording prior to induction of FEA. The trace in (B) was recorded 5 min following the start of PG ejection (69 kPa). The trace (C) is a continuation of (B) demonstrating that the application of a mini-gator clip to the rat's tail (TC) suppresses FEA during and briefly following its removal.


A. Control
B. PG 69 kPa
C. Response to Noxious Stimulation
TC
350 µV
10 sec

[^{125}I] cyanopindolol (Pazos et. al., 1987). Activating this receptor decreases 5-HT release from raphe neuron terminals (Pettibone and Pflueger, 1984; Chaput et. al., 1986).

If 5-HT mediates the suppression of FEA by NS, 5-HT agonists with affinity for the 5-HT$_{1A}$ somatic autoreceptor should block the suppression of FEA, provided that 1) the activity of raphe neurons is important in the process and 2) 5-HT$_{1A}$ receptors don't mediate the suppression. Activating the terminal autoreceptor should also block the suppression of FEA by NS, by reducing 5-HT release from raphe neuron terminals.

The prototypic 5-HT$_{1A}$ agonist 8-OH-DPAT (Hjorth et. al., 1982; Leysen, 1985) (0.9-1.5 µmol/kg i.v., n=5) blocked the suppression of FEA by NS within 1 min of administration (Fig 5). The average duration of the blockade of the suppression of FEA by NS was 46 min (Table 1). 2-MPP (0.4-4.4 µmol/kg i.v., n=4), with affinity for both 5-HT$_{1A}$ and 5-HT$_{1B}$ binding sites (Hamon et. al., 1986), blocked the suppression of FEA by NS within 2.5-5 min of administration (Fig 6). The average duration of the blockade was 61 min (Table 1). The 5-HT$_{1B}$ agonist TFMPP (Hamon et. al., 1986) (0.4-4.4 µmol/kg, i.v.) blocked the suppression of FEA by NS (Fig 7) within 30 sec-4 min of administration. The average duration of the suppression of FEA by NS was 43 min (Table 1).

3.3 The Effect of Amine Uptake Blockers on the Suppression of FEA by NS

3.31 Desimipramine (DMI) and Imipramine (IMI) and the Suppression of FEA by NS

Figure 5. The i.v. administration of 8-OH-DPAT blocked the suppression of FEA by NS in the rat. Control response (A) shows the suppression of FEA by TC.. Administration of 8-OH-DPAT blocked the suppression of FEA by NS (B). Recovery was apparent within 31 min (C). The administration of 8-OH-DPAT again blocked the suppression of FEA by NS (D) while QPZ, administered 4 min later, restored the suppression of FEA by NS and reduced the background level of FEA (E).

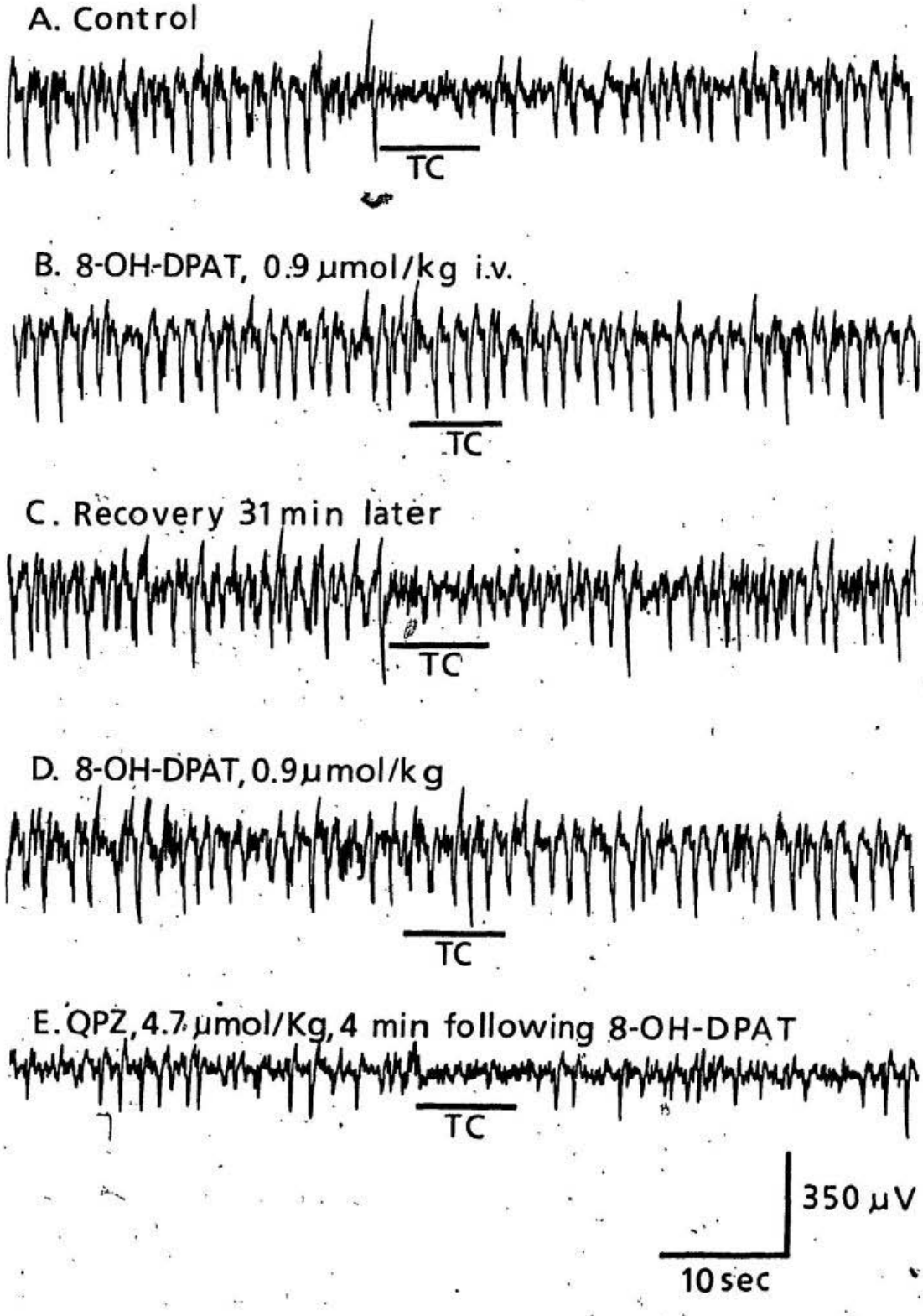

A. Control
TC
B. 8-OH-DPAT, 0.9 µmol/kg i.v.
TC
C. Recovery 31 min later
TC
D. 8-OH-DPAT, 0.9 µmol/kg
TC
E. QPZ, 4.7 µmol/Kg, 4 min following 8-OH-DPAT
TC
350 µV
10 sec

Table 1. The change in background level of FEA and the duration of the blockade of the suppression of FEA by NS following 8-OH-DPAT, 2-MPP and TFMPP.

Drug (µmol/kg)	Rat#	Change in FEA (% of control)	Duration (min)
8-OH-DPAT			
0.9	1	104	30
0.9	2	82*	83
0.9	3	98	30
1.2	4	95	51
1.5	5	102	35
2-MPP			
0.4	1	89*	95
0.4	2	94	69
2.2	3	97	45
4.4	4	48*	35
TFMPP			
0.4	1	104	25
0.4	2	102	63
2.2	3	86*	30
4.4	4	84*	55

*=Significant P< 0.05

Figure 6. The i.v. administration of 2-MPP blocked the suppression of FEA by NS in the rat. Control response (A) shows the suppression of FEA by TC. Administration of 2-MPP blocked the suppression of FEA by NS (B). Recovery was apparent within 98 min (C).

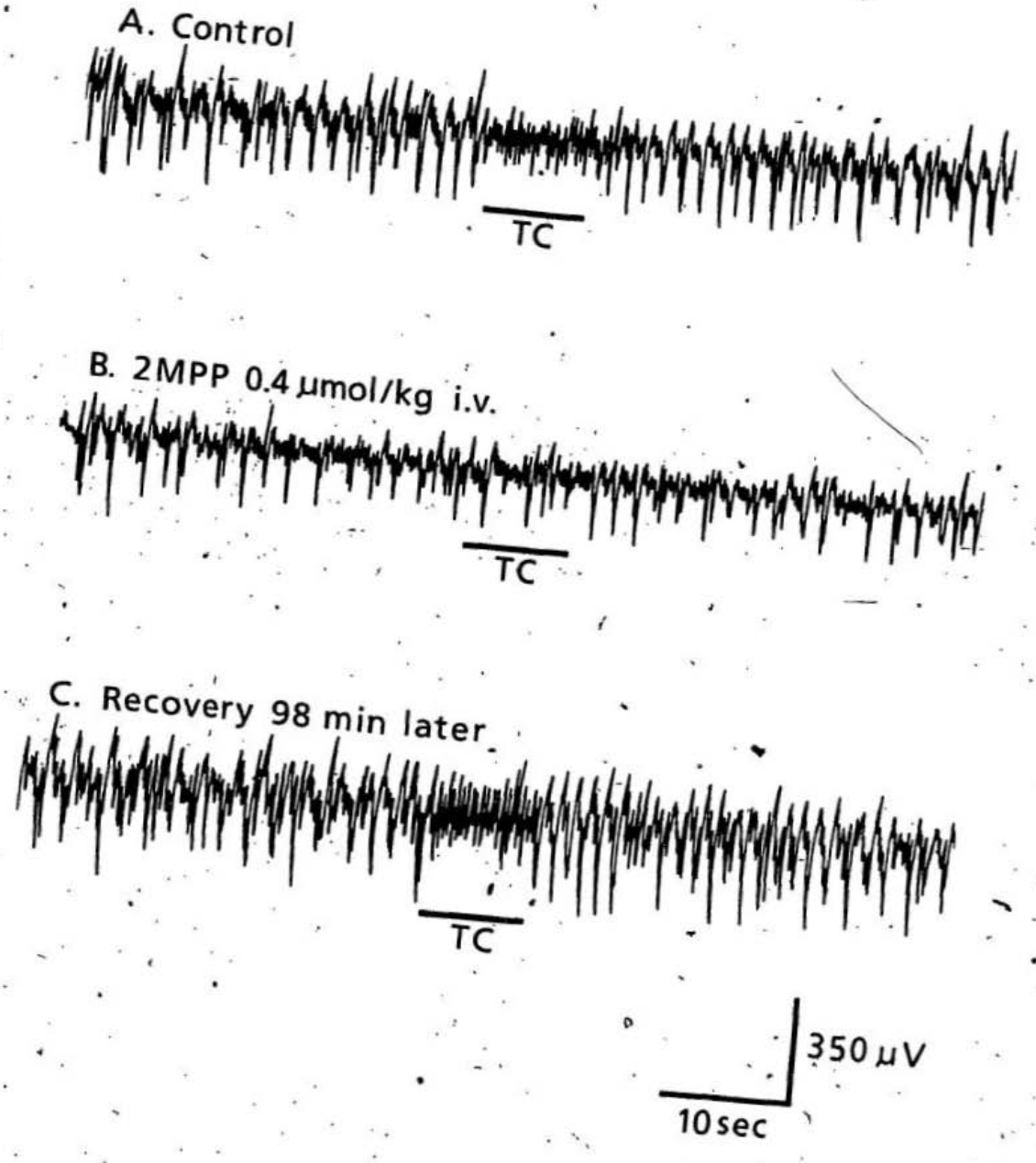
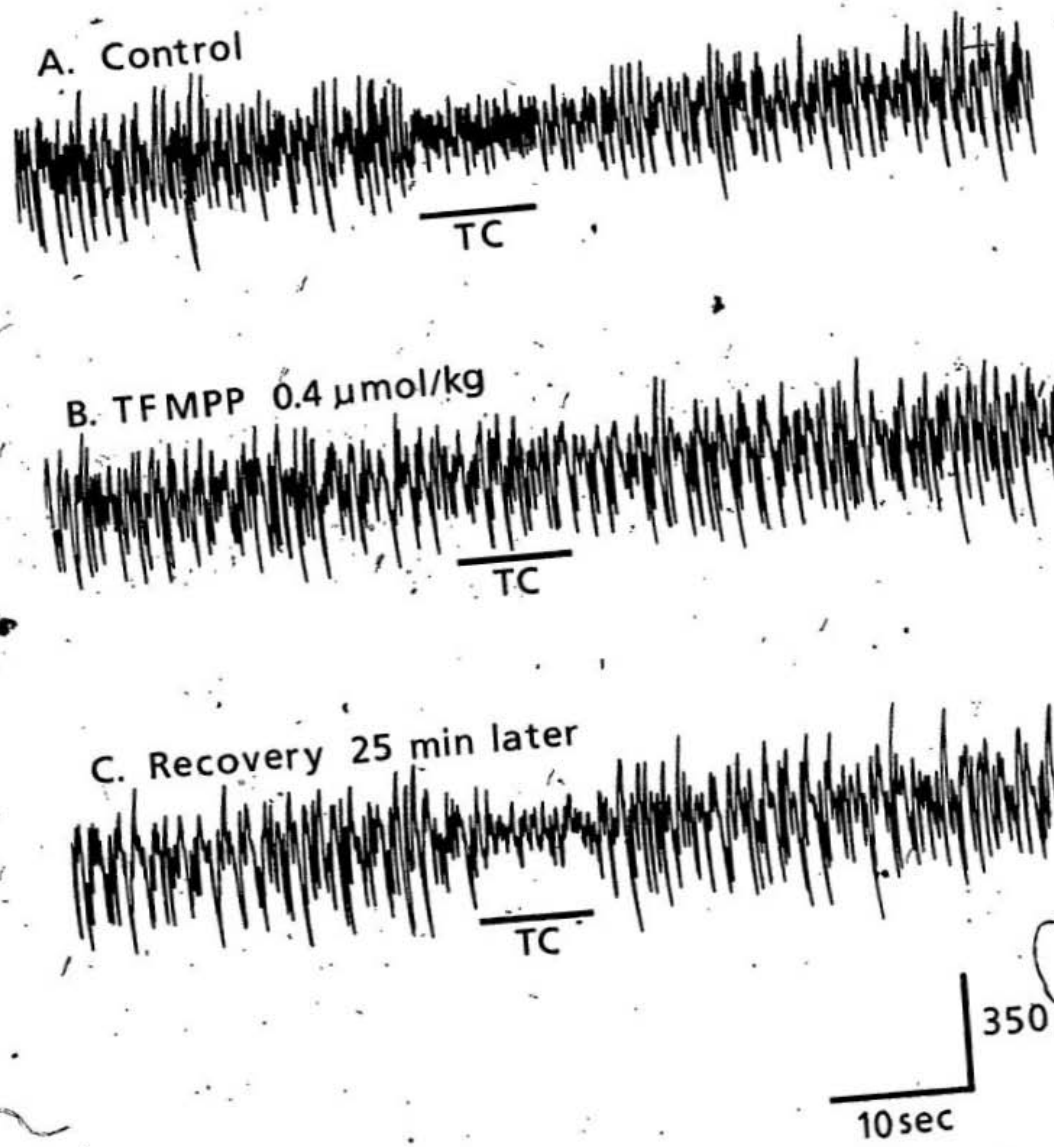

A. Control
TC
B. 2MPP 0.4 µmol/kg i.v.
TC
C. Recovery 98 min later
TC
350 µV
10 sec

Figure 7. The i.v. administration of TFMPP blocked the suppression of FEA by NS in the rat. Control response (A) shows the suppression of FEA by TC. Administration of TFMPP blocked the suppression of FEA by NS. Recovery was apparent within 25 min (C).


A. Control
TC
B. TFMPP 0.4 µmol/kg
TC
C. Recovery 25 min later
TC
350 µV
10 sec

Another way to activate the 5-HT somatic autoreceptor, and thus inhibit unit activity, is to increase the concentration of 5-HT in the vicinity of the autoreceptor. This can be accomplished by blocking the uptake of 5-HT using IMI. IMI, however, also blocks the uptake of NE and therefore inhibits locus coeruleus (LC) unit activity (Nyback et. al., 1975). To test for possible effects of reducing LC unit activity on the response to IMI, DMI was administered prior to IMI. DMI selectively blocks the uptake of NE, but not 5-HT, leading to the inhibition of LC but not dorsal or median raphe neurons (Sheard et. al., 1972).

The administration of DMI (10 µmol/kg i.v., n=9) did not reduce the effectiveness of NS in suppressing FEA, although it caused a statistically significant reduction in the background level of FEA in 8 of 9 animals tested (Table 2). Thus it appears that LC unit activity is not essential for the suppression of FEA by NS. However, when IMI (11 µmol/kg i.v., n=7) was administered following DMI it blocked the suppression of FEA by NS (Fig 8) and caused an increase in the background level of FEA in all rats tested. IMI administered alone (n=3) also blocked the suppression of FEA by NS demonstrating that DMI did not influence the outcome with IMI.

3.32 Fluoxetine (FLU)

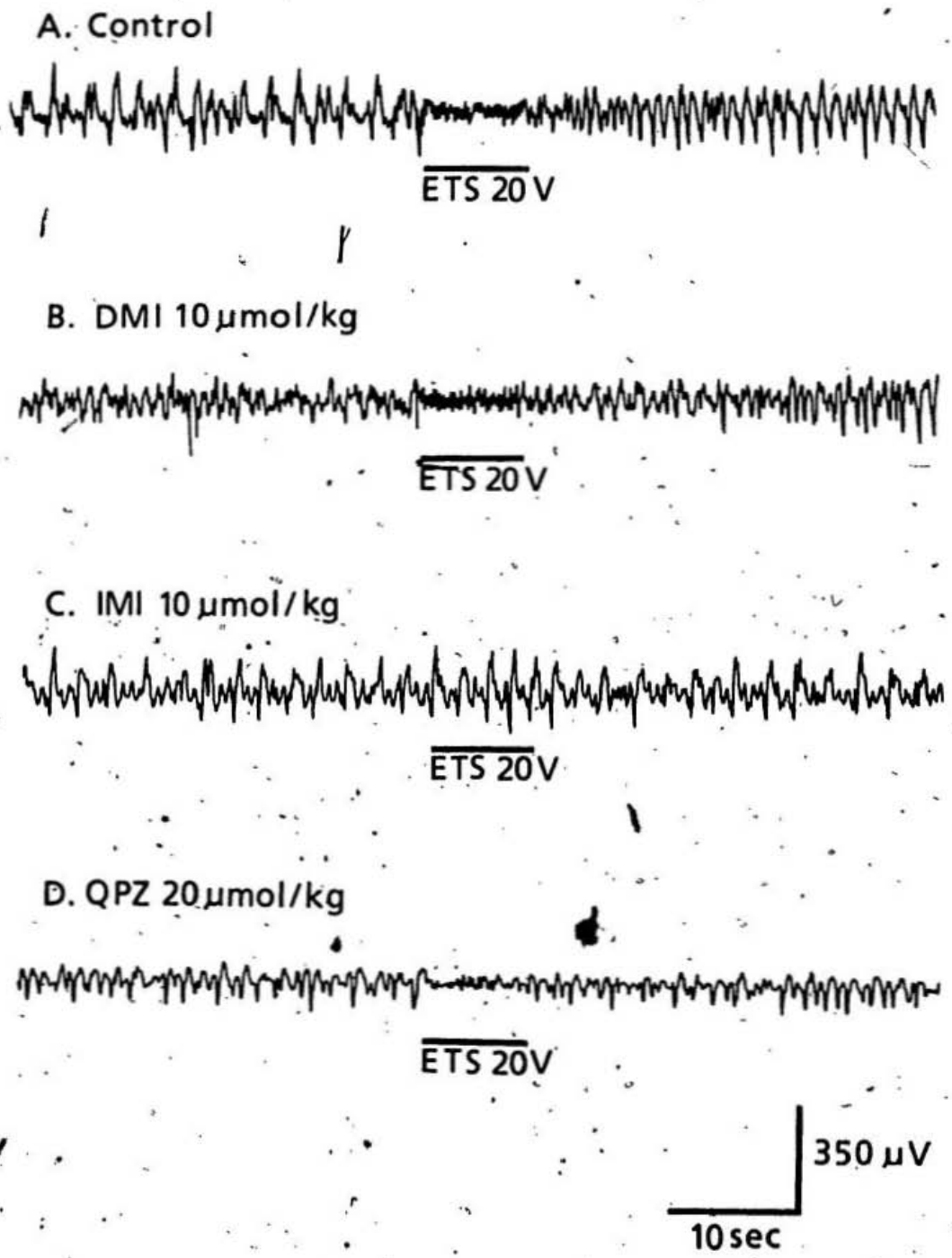
In contrast to IMI, FLU is a specific blocker of 5-HT uptake with little or no effect on the uptake of dopamine or

Table 2. The change in background level of FEA following the administration of IMI, DMI, and QPZ.

Drug	Rat#	Change in FEA (% of control)
DMI	1	103
11 µmol/kg	2	56*
	3	39*
	4	78*
	5	64*
	6	69*
	7	42*
	8	66*
	9	63*
IMI	1	108
11 µmol/kg	2	Not Tested
	3	135*
	4	109
	5	94
	6	Not Tested
	7	85*
	8	153*
	9	117
QPZ	1	52*
14 µmol/kg	2	Not Tested
	3	Not Tested
	4	Not Tested
	5	62*
	6	Not Tested
	7	99
	8	48*
	9	84

*=Significant P< 0.05

Figure 8. The effect of DMI, IMI and QPZ on the suppression of FEA by NS in the rat. DMI failed to alter the suppression of FEA by NS (B) compared to control (A), but did reduce the background level of FEA. IMI administered following DMI blocked the suppression of FEA by NS (C). QPZ administered shortly after record in (C) restored the suppression of FEA by NS and reduced the background level of FEA (D).


A. Control
ETS 20 V
B. DMI 10 µmol/kg
ETS 20 V
C. IMI 10 µmol/kg
ETS 20 V
D. QPZ 20 µmol/kg
ETS 20 V
350 µV
10 sec

norepinephrine (De Montigny and Aghajanian, 1978). As with IMI, FLU (3 µmol/kg i.v., n=4) blocked the suppression of FEA by NS (Fig 9). The duration of action was quite brief for both doses of FLU (Table 3).

3.4 The Effect of Quipazine (QPZ) on the Suppression of FEA by NS

QPZ antagonizes some actions of 8-OH-DPAT (Goodwin and Green, 1985). QPZ was administered following the blockade of the suppression of FEA by 8-OH-DPAT to test for possible antagonism. QPZ (4.7-14 µmol/kg i.v., n=3) restored the suppression of FEA by NS (Fig 5) in 2 of 3 rats tested (Table 4).

QPZ was also used to attempt to antagonize the blockade of the suppression of FEA by NS induced by IMI. It was felt that QPZ might restore the suppression of FEA by NS, since IMI was thought to indirectly inhibit raphe firing. QPZ (14 µmol/kg i.v., n=5) restored the suppression of FEA by NS (Fig 8) in all animals tested (Table 4). The administration of QPZ (4.7-23 µmol/kg i.v., n=4) to untreated rats significantly decreased the background level of FEA, but did not alter the suppression of FEA by NS.

3.5 The Pressure Ejection of 5-HT and 8-OH-DPAT Into the Dorsal Raphe

IMI, FLU, 8-OH-DPAT and TFMPP have been shown to decrease raphe firing (Sheard et. al., 1972; Trulson and

Figure 9. Fluoxetine blocked the suppression of FEA by NS in the rat. Control response (A) shows the suppression of FEA by TC. Administration of fluoxetine blocked the suppression of FEA by NS (B). Recovery was apparent within 14 min (C).

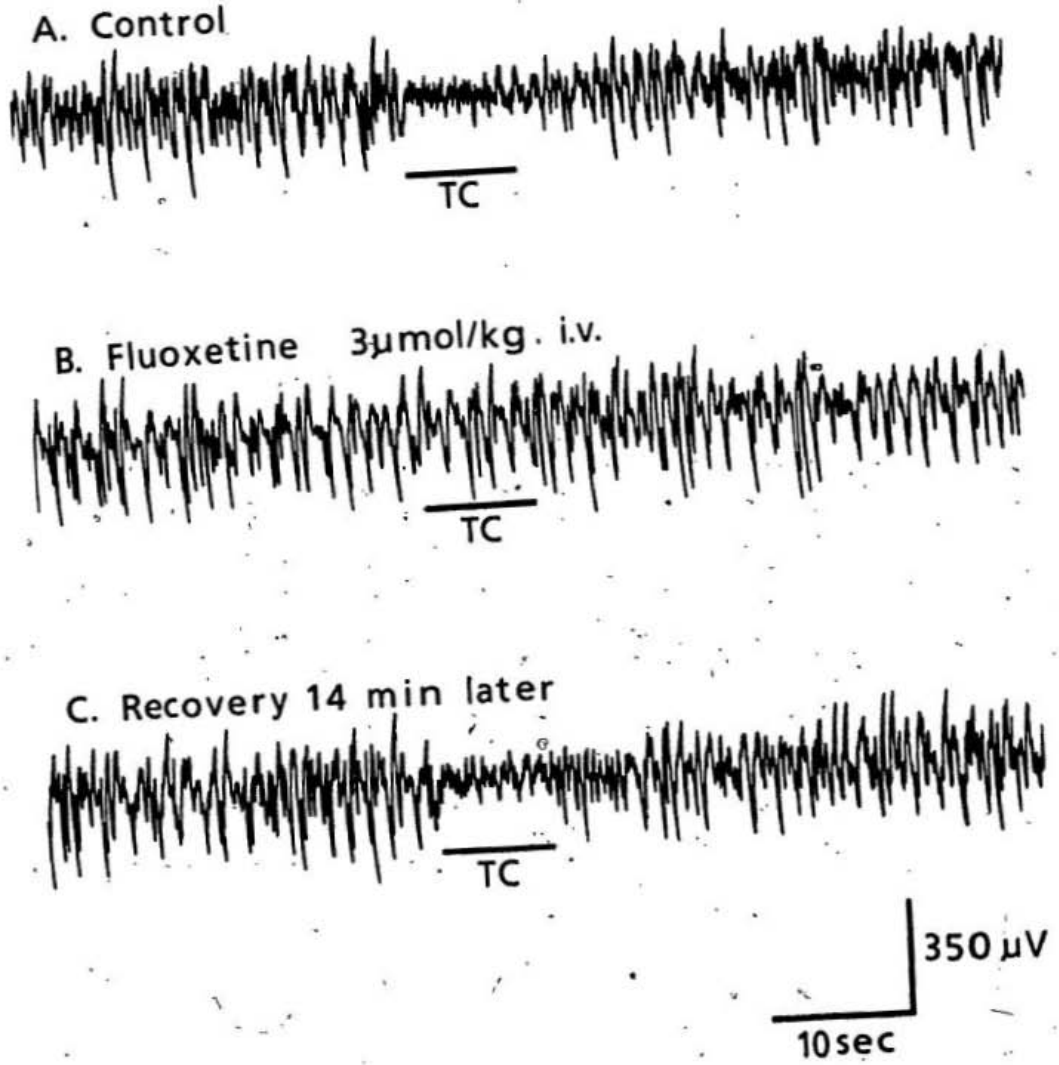

A. Control
TC
B. Fluoxetine 3µmol/kg . i.v.
TC
C. Recovery 14 min later
TC
350 µV
10sec

Table 3. The change in background level of FEA and duration of the blockade of the suppression of FEA by NS following FLU.

Drug	Rat#	Change in FEA (% of control)	Duration (min)
FLU	1	102	11
3 µmol/kg	2	106	15
	3	71*	11
	4	63*	18
FLU	1	103	20
16 µmol/kg	2	101	12
	3	74*	16
	4	Not Tested	

*=Significant P< 0.05

Table 4. The effect of QPZ on the suppression of FEA by NS in 8-OH-DPAT pretreated rats, DMI and IMI pretreated rats and normal rats.

Drug (μmol/kg)	Rat#	Change in FEA (% of control)	Restored Suppression
QPZ (Following 8-OH-DPAT)			
4.7	1	86*	No
14	1	88*	No
4.7	2	75*	Yes
4.7	3	88*	Yes
	4	Not Tested	
	5	Not Tested	

Drug (μmol/kg)	Rat#	Change in FEA (% of control)	Restored Suppression
QPZ (Following DMI and IMI)			
14	1	52*	Yes
14	2	Not Tested	
14	3	Not Tested	
14	4	Not Tested	
14	5	62*	Yes
14	6	Not Tested	
14	7	99	Yes
14	8	48*	Yes
14	9	84	Yes

Drug (μmol/kg)	Rat#	Change in FEA (% of control)	Duration (min)	Blocked Suppression
QPZ (Untreated Rats)				
4.7	1	68*	12	No
4.7	2	83*	16	No
14	2	82*	15	No
4.7	3	79*	10	No
14	3	79*	9	No
23	3	79*	7	No
4.7	4	79*	10	No
14	4	88*	9	No
23	4	94*	5	No

*=Significant P< 0.05

Crisp, 1976; Sprouse and Aghajanian, 1985; Sprouse and Aghajanian, 1986). All of these agents blocked the suppression of FEA by NS when administered i.v. Although those observations suggest involvement of the raphe nuclei in mediating the suppression of FEA, other interpretations are possible. However, involvement of the dorsal raphe could be directly tested by local drug application. Clearly if dorsal raphe firing is necessary for the suppression of FEA by NS, then the local pressure ejection of 5-HT or 8-OH-DPAT into the dorsal raphe region should activate the somatic autoreceptors leading to a decrease in raphe firing and, hence, block the suppression of FEA by NS. Furthermore, the pressure ejection of the excitatory neurotransmitter glutamate (GLU) into the same region might be expected to restore the suppression of FEA by NS by directly exciting raphe neurons.

3.51 5-HT

The pressure ejection of 100 mM 5-HT (100-1000 msec; 280 kPa [n=7]) blocked the suppression of FEA by NS in all rats tested (Table 5). Lower concentrations of 5-HT (1 or 10 mM) were ineffective (n=6). The effectiveness of 5-HT and 0.2 M GLU varied within each rat depending on the depth of electrode placement. The average interval between pressure ejection of 100 mM 5-HT and blockade of the suppression of FEA by NS was 6.6 ± 3.9 min. Although tissue damage in the area of the dorsal raphe during sectioning prevented the

Table 5. The response obtained following the pressure ejection of 100 mM 5-HT at various depths in the region of the dorsal raphe.

Drug	Rat#	Depth Tested (mm)	Pulse Duration (msec)	Suppression Blocked Time (min)	.2 M GLU Reversal	Electrode in Raphe?
5-HT						
100 mM	1	-6.5	750	No	NT	Yes
100 mM	1	-6.8	950	Yes 10	Yes	Yes
100 mM	1	-7.1	550	No	NT	Yes
100 mM	2	-6.5	750	No	NT	Yes
100 mM	2	-6.8	100	Yes 3	NT	Yes
100 mM	3	-6.5	750	No	NT	Yes
100 mM	3	-6.7	850	Yes 12	Yes	Yes
100 mM	3	-7.5	600	Yes 2	No	Yes
100 mM	4	-6.8	325	Yes 5.5	Yes	NHA
100 mM	5	-6.5	1000	Yes 1.5	NT	NHA
100 mM	6	-6.0	350	Yes 10	NT	NHA
100 mM	6	-6.5	350	Yes 10	Yes	NHA
100 mM	6	-7.0	100	Yes 4.5	Yes	NHA
100 mM	7	-6.5	850	Partial 6.5	Yes	NHA
100 mM	7	-7.0	750	No	NT	NHA

NHA=No Histology Available
NT=Not Tested

determination of electrode placements relative to the dorsal raphe in 4 of 7 rats, the average interval between pressure ejection of drug and blockade of the suppression of FEA by NS was similar for animals with (7 ± 5.3 min) and without histological varification of electrode placements (6.3 ± 3.3 min).

3.52 8-OH-DPAT

Both 1 and 10 mM 8-OH-DPAT were effective at some sites in blocking the suppression of FEA by NS (Table 6). As was the case with the pressure ejection of 5-HT, the effectiveness of 8-OH-DPAT and GLU varied within each animal depending on the depth of electrode placement. In one rat for which histology verified that the electrode was positioned in the dorsal raphe region, 1 mM 8-OH-DPAT failed to block the suppression of FEA by NS. This may indicate that a higher concentration of 8-OH-DPAT is necessary to cause a blockade. The average interval between pressure ejection of 8-OH-DPAT and blockade of the suppression of FEA by NS was 9.2 ± 4.8 min.

Increasing the concentration of 8-OH-DPAT in the micropipette to 100 mM resulted in more consistent blockade of the suppression of FEA (Table 7). The average interval between pressure ejection of 100 mM 8-OH-DPAT and blockade of the suppression of FEA by NS was 4.25 ± 3.2 min. Tissue rippage prevented the determination of the position of electrode tracks relative to the dorsal raphe in the 3 rats tested. Based on the available histology, it is clear that

Table 6. The response obtained following the pressure ejection of 1 and 10 mM 8-OH-DPAT at various depths in the region of the dorsal raphe.

Drug	Rat#	Depth Tested (mm)	Pulse Duration (msec)	Suppression Blocked Time (min)	.2 M GLU Reversal	Electrode in Raphe?
8-OH-DPAT						
1 mM	1	-6.5	2200	Partial 5.5	NT	NHA
1 mM	1	-6.7	3700	Yes 16	Yes	NHA
1 mM	2	-6.5	1950	No	NT	Yes
1 mM	2	-6.7	2800	No	NT	Yes
1 mM	2	-6.9	2100	No	NT	Yes
10 mM	3	-6.5	3700	No	NT	NHA
10 mM	3	-6.7	2550	No	NT	NHA
10 mM	3	-6.9	1100	Yes 14	Yes	NHA
10 mM	4	-6.5	2800	No	NT	NHA
10 mM	4	-6.7	1000	No	NT	NHA
10 mM	4	-7.0	1500	No	NT	NHA
10 mM	5	-6.7	2000	No	NT	Yes
10 mM	5	-7.0	2000	Yes 5	Yes	Yes
10 mM	5	-7.3	1800	Yes 15	No	Yes
10 mM	5	-7.5	3900	No	NT	Yes
10 mM	6	-6.7	200	Yes 4	Yes	NHA
10 mM	6	-6.9	2900	No	NT	NHA
10 mM	7	-6.7	900	Yes 11	NT	NHA
10 mM	7	-7.0	1800	Yes 7.5	Partial	Yes
10 mM	7	-7.5	700	Yes 5	Yes	Yes

NHA=No Histology Available
NT=Not Tested

Table 7. The response obtained following the pressure ejection of 100 mM 8-OH-DPAT at various depths in the dorsal raphe.

Drug	Rat#	Depth Tested (mm)	Pulse Duration (msec)	Suppression Blocked	Time (min)	.2 M GLU Reversal	Electrode in Raphe?
8-OH-DPAT							
100 mM	1	-6.8	400	Yes	1.5	Yes	NHA
100 mM	1	-7.0	200	Yes	1.5	No	NHA
100 mM	1	-7.2	200	Yes	3	No	NHA
100 mM	2	-6.7(S1)	425	No		NT	NHA
100 mM	2	-7.0(S1)	675	No		NT	NHA
100 mM	2	-7.5(S1)	1400	No		NT	NHA
100 mM	2	-6.7(S2)	750	Yes	5.5	Yes	NHA
100 mM	2	-7.0(S2)	1100	No		NT	NHA
100 mM	3	-6.0	750	No		NT	NHA
100 mM	3	-6.5	200	Yes	10	Yes	NHA
100 mM	3	-7.0	750	Yes	4	Yes	NHA

NHA=No Histology Available
NT=Not Tested
S1=Site 1
S2=Site 2

in the majority of responsive sites tested in which the pressure ejection of 5-HT or 8-OH-DPAT blocked the suppression of FEA by NS, the micropipette was positioned in the dorsal raphe region. In the majority of non-responsive sites tested the micropipette was found to be positioned outside the dorsal raphe region (Fig 10).

3.6 Intracortical Application of 5-hydroxytryptophan (5-HTP) in PCPA Treated Rats

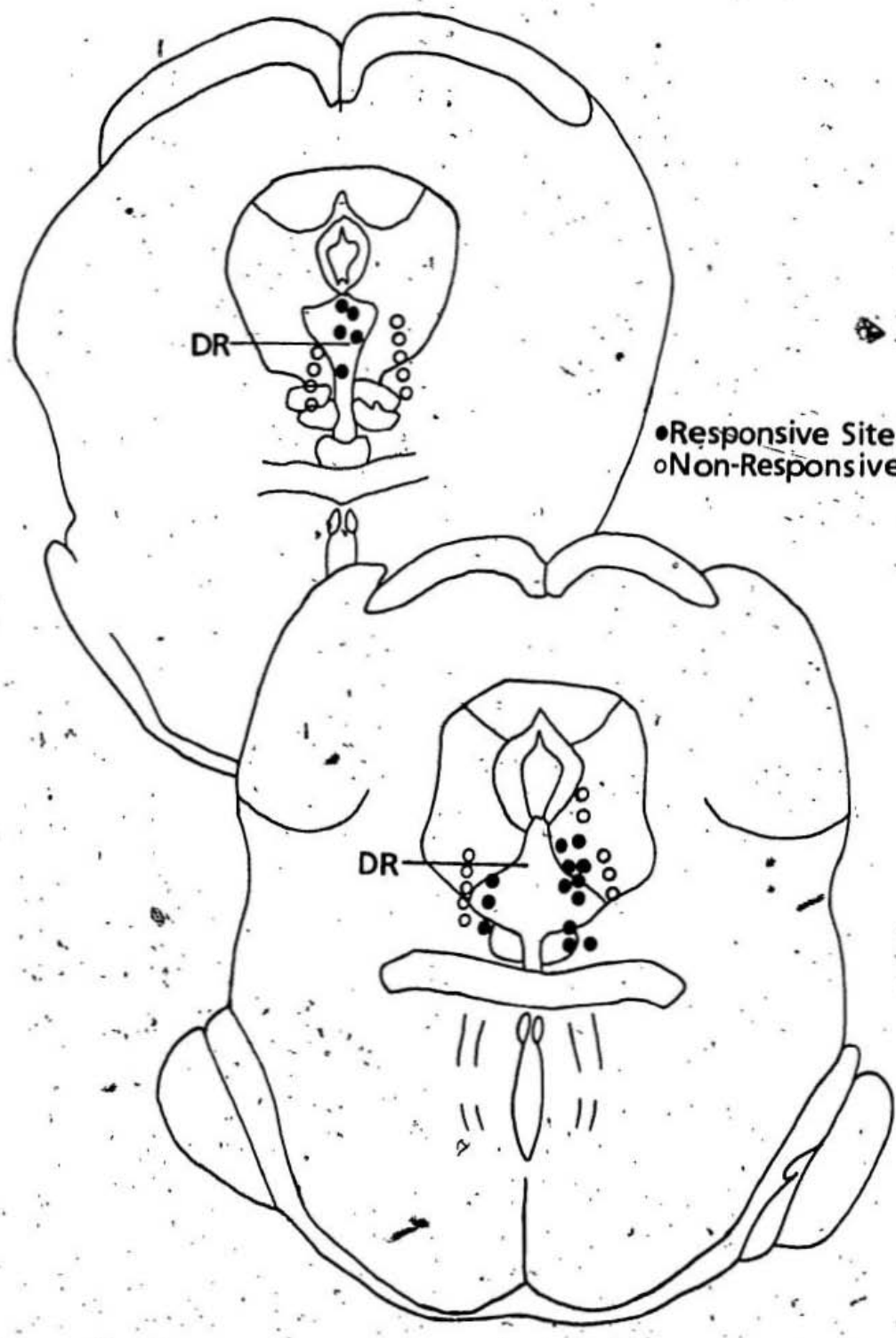
3.61 Effects of 5-HTP on FEA

In PCPA treated animals, NS is ineffective in suppressing FEA (Neuman, 1986b). As the rostral raphe nuclei appear to be the most sensitive to autoinhibition (Haigler and Aghajanian, 1974) and these project to the forebrain, it suggests that forebrain structures (O'Hearn and Molliver, 1984) may be important in mediating the suppression of FEA by NS. Since 5-HT is released cortically and FEA is induced cortically, this suggests that 5-HT might act cortically to reduce FEA. To test this hypothesis, 5-HTP was pressure ejected intracortically, at the same site as PG application, in PCPA treated animals in an attempt to restore 5-HT transmission selectively at this level and thereby restore the suppression of FEA by NS.

The rationale for these experiments was that since PCPA does not reduce the activity of raphe neurons but rather only reduces the level of neuronal 5-HT, if 5-HT levels could be restored at the site of action, the suppression of FEA by NS

Figure 10. Histological localization of the micropipette tracks in the vicinity of the dorsal raphe (DR). The majority of responsive sites were found to be located in the DR while the majority of non-responsive sites were found to be located outside DR.

AP 7.3-7.8



could also be restored.

The local application of 65 mM 5-HTP (0.33-44 min; 34-128 kPa) to 4 rats pretreated with PCPA caused a statistically significant decrease in the background level of FEA in all rats tested. Furthermore in 2 of the 4 rats the pressure ejection of 5-HTP restored the suppression of FEA by NS (Table 8).

3.62 Immunohistochemistry

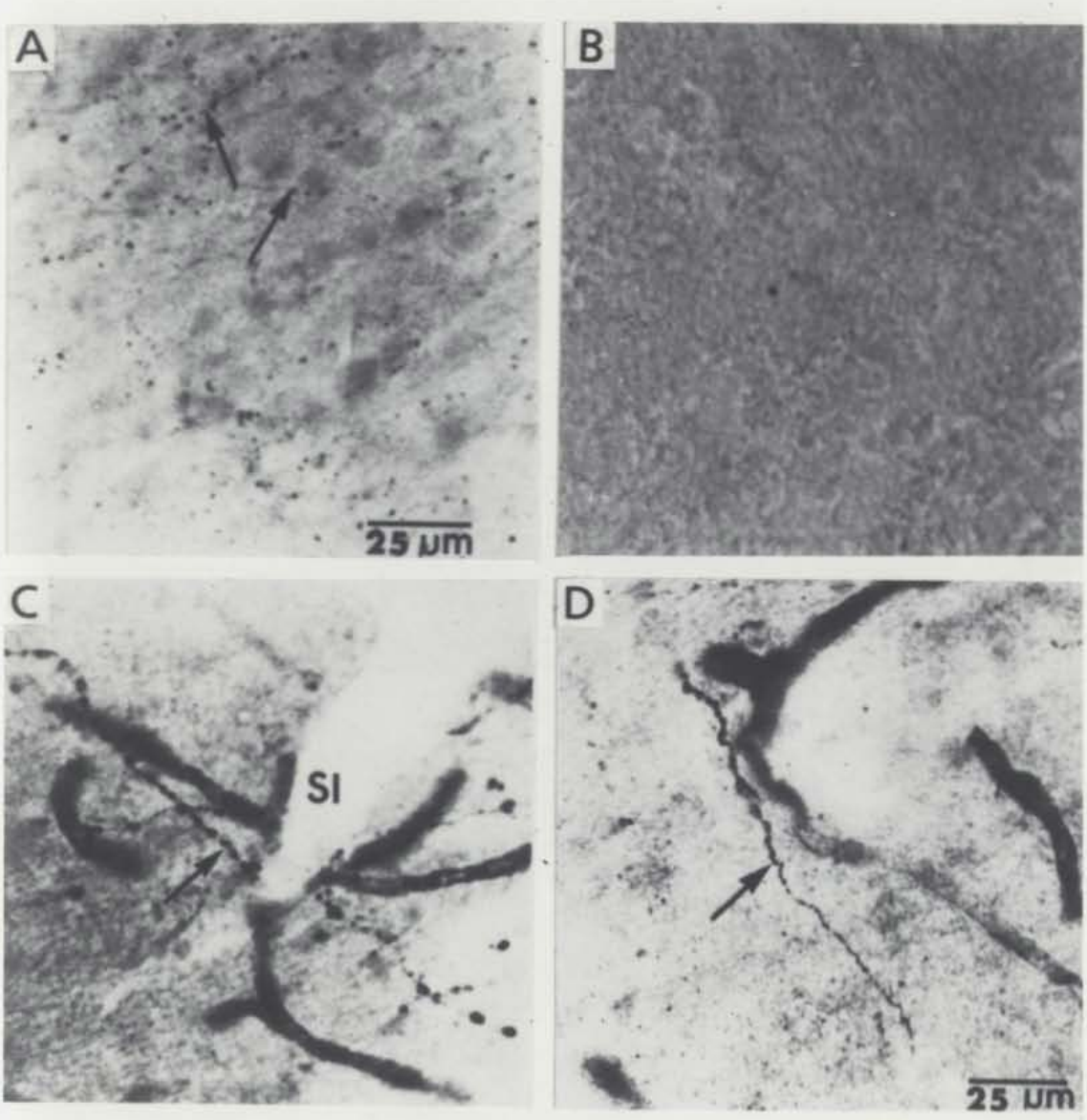
PCPA treated brain tissue processed using the PAP method was essentially devoid of neuronal processes showing 5-HT-like immunoreactivity (5-HTLI), which stained dark brown in controls, with the exception of two regions: 1) the area in the left hemisphere of the cerebral cortex into which 5-HTP had been pressure ejected 2) the decussation of the ventral tegmentum. The remaining regions of the brain tissue were observed to be a pale yellow shade, indicative of an absence of 5-HTLI. In the area of the cortex into which 5-HTP had been pressure ejected, many blood capillaries were observed to have stained positive for 5-HTLI. Processes containing 5-HTLI could be detected scattered among the stained capillaries. The majority of the processes observed were located in close proximity to the site of injection of 5-HTP. Numerous small, round varicosities (0.8-1.4 µm in diameter) were clearly visible on all neuronal processes staining positive for 5-HTLI. In untreated rats numerous neuronal processes staining positive for 5-HTLI were found

Table 8. The duration of the change of background level of FEA following the intracortical pressure ejection of 5-HTP in PCPA treated rats.

kPa	min	Rat#	Change in FEA (% of Control) After 5-HTP	Restored Suppression	Duration (min)
69	0.33	1	26.1*	No	12
138	1.5	2	73.8*	Yes	17
138	7	3	58.4*	No	18
138	44	4	87.5*	Yes	28

* Significant $P < 0.05$

Figure 11. (A) Neuronal processes (some indicated by arrows) staining positive for 5-HT like immunoreactivity (5-HTLI) in the cerebral cortex of untreated rats. (B) The same area of the cerebral cortex in PCPA treated rats did not show neuronal processes staining positive for 5-HTLI. (C) Neuronal process (indicated by arrow) and blood capillaries containing 5-HTLI in the cerebral cortex of a PCPA treated rat located proximal to the site of pressure ejection of 5-HTP (SI) from a micropipette. (D) Neuronal process (indicated by arrow) and blood capillaries containing 5-HTLI below SI in another PCPA treated rat.


A
25 µm
B
C
SI
D
25 µm

throughout the entire cerebral cortex (Fig 11).

In the region of the decussation of the ventral tegmentum, a small number of fibres staining positive for 5-HTLI were observed with varicosities 0.6-1.2 µm in diameter. The few fibres visualized were of a much shorter length than those observed in the cortex in the area where 5-HTP had been pressure ejected. In untreated rats the region of the decussation of the ventral tegmentum was found to contain many fibres showing 5-HTLI. Fibres were of a much greater length than those observed in the equivalent region of PCPA treated rats with varicosities 0.6-1.2 µm in diameter and intervaricose connections clearly visible.

3.7 Intracortical Application of 5-HT and Drugs Releasing 5-HT

3.71 Intracortical Pressure Ejection of 5-HT

Since the local pressure ejection of 5-HTP intracortically in PCPA treated rats provided some suggestive evidence that an increase in 5-HT at the cortical level might mediate the suppression of FEA by NS, attempts were made to simulate the effects of NS by the local pressure ejection 5-HT. The pressure ejection of 1 mM 5-HT (100-800 msec pulses; 550 kPa [n=2]) had no effect upon the amplitude of FEA or upon the duration of suppression of FEA in response to NS. Increasing the concentration of 5-HT ejected (10 mM, [n=4]) was also found to be without effect on FEA.

3.72 Intracortical Application of p-chloroamphetamine (PCA)

5-HT is likely to be rapidly transported into aminergic terminals and/or metabolized when applied by pressure ejection. As a result, the local concentration or spread of 5-HT may be insufficient to have a demonstratable effect on FEA. PCA, which releases 5-HT as well as inhibiting its reuptake and synthesis (Sanders-Bush and Steranka, 1978), was applied cortically by pressure ejection as an alternative test of the importance of the cortical site of action of 5-HT.

The pressure ejection of 10 mM PCA (100-800 msec pulses; 550 kPa [n=4]) resulted in a suppression of FEA that was not duplicated by pressure ejection of vehicle at the same pressure. The suppression of FEA consisted of a statistically significant reduction in the background level of FEA in each rat tested with the average reduction in the background level of FEA being 77.5 ± 10.2% of control (Table 9). The response to NS following recovery of the background level of FEA was unaltered from the control response to NS, with the exception of 1 animal in which the suppression was slightly prolonged. Once a response to PCA had been obtained, the further ejection of PCA was without effect.

3.73 The Pressure Ejection of Fenfluramine (FEN)

FEN, like PCA, also releases 5-HT (Zemlan, 1978). However, the pressure ejection of 10 mM FEN (100-800 msec pulses; 550 kPa [n=4]) failed to suppress the background level of FEA or facilatate the suppression of FEA by NS.

Table 9. The duration of the change of background level of FEA following the intracortical pressure ejection of PCA.

Rat#	Pulse Duration (msec)	Change in FEA (% of control)	Duration (min)
1	250	73.9*	3
2	500	74.5*	8
3	500	92.4*	4
4	800	69.3*	5

* Significant $P < 0.05$

Increasing the concentration of FEN ejected (20 and 100 mM) was also ineffective (n=2).

3.8 Baclofen and the Suppression of FEA by NS

Baclofen, a $GABA_B$ agonist, reduces the cortical release of 5-HT (Schlicker et. al., 1984; Gray et. al., 1986). Since it was hypothesized that 5-HT might be acting cortically to mediate the suppression of FEA by NS, reducing the cortical release of 5-HT with baclofen should block the suppression of FEA by NS.

Baclofen (4.7-9.4 µmol/kg i.v., n=4) blocked the suppression of FEA by NS in all rats tested (Fig 12). Recovery of the response was not apparent within 1-2 hrs following injection (duration of recording). Baclofen caused a statistically significant increase in the background level of FEA in 2 of the 5 rats tested (Table 10). Although baclofen has a potent analgesic action (Cutting and Jordan, 1975; Levy and Proudfit, 1977), leg withdrawal in response to NS applied to the hindpaw was still present even though the suppression of FEA by NS was blocked by baclofen.

3.9 Identification of the 5-HT Receptor Subtype Mediating the Suppression of FEA by NS

If 5-HT mediates the suppression of FEA, then by using various antagonists it should be possible to identify the 5-HT receptor subtype involved in the process.

Figure 12. Baclofen blocked the suppression of FEA by NS. Control response (A) shows suppression of FEA by TC. The i.v. administration of baclofen blocked the suppression of FEA by NS (B). Recovery was not apparent within 1-2 hrs.

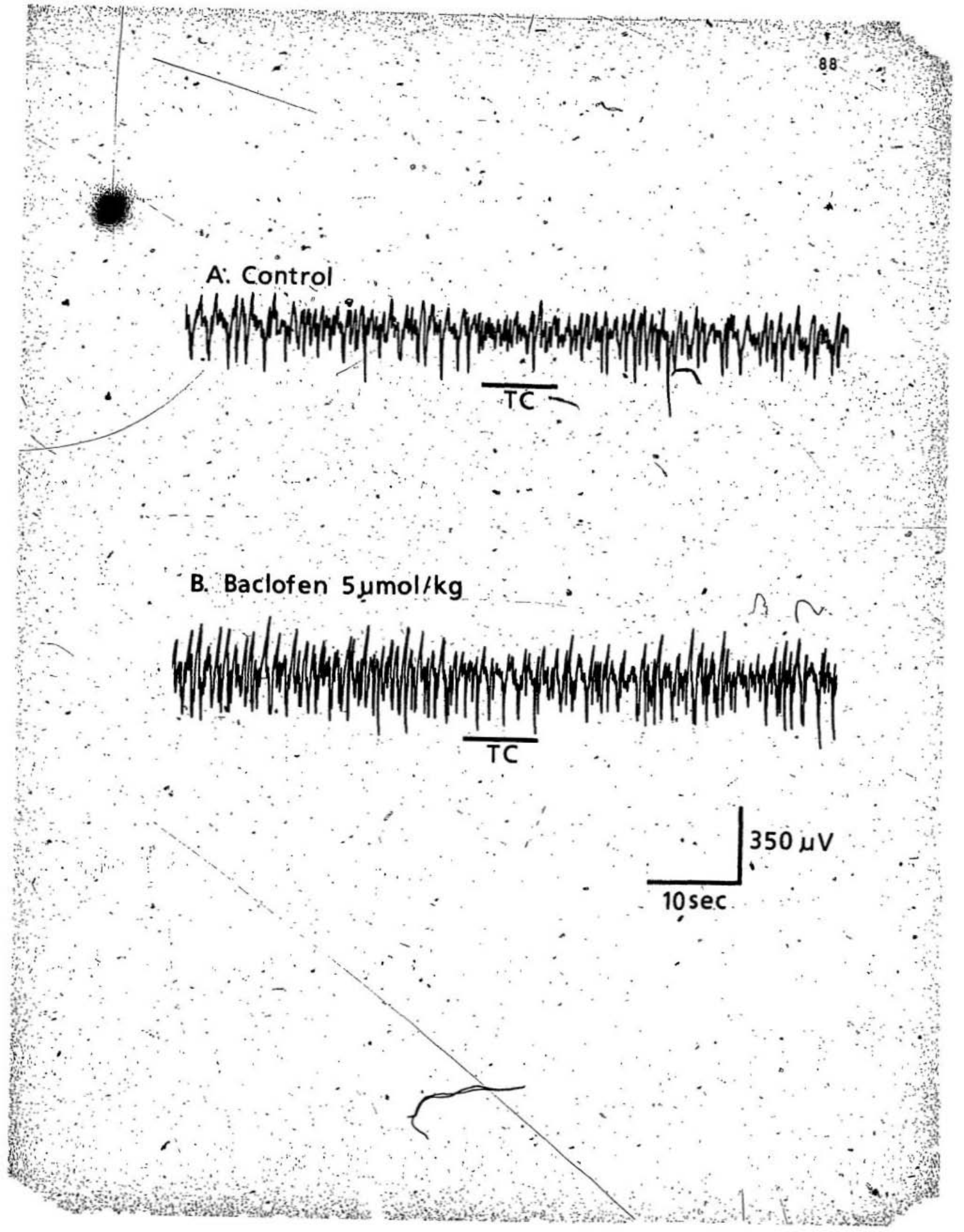

A. Control
TC
B. Baclofen 5µmol/kg
TC
350 µV
10sec

Table 10. The change of background level of FEA following the i.v. administration of Baclofen.

Drug (µmol/kg)	Rat#	Change in FEA (% of control)
Baclofen		
4.7	1	123*
4.7	2	130*
4.7	3	115
9.4	4	103
9.4	5	101

*=Significant P< 0.05

Antagonists with affinity for either 5-HT_1, 5-HT_2 or 5-HT_3 binding sites were tested for their ability to block the suppression of FEA by NS. The results of these experiments are shown in Table 11. It appears unlikely that 5-HT_3 receptors are involved as ICS 205 930 was ineffective in antagonizing the suppression of FEA by NS. On the other hand, the antagonists which were effective all bind to 5-HT_2 and/or 5-HT_{1C} receptors. However, even this is not uniform as neither metergoline or methysergide were effective despite the fact they bind to both 5-HT_{1C} and 5-HT_2 receptors (Heuring and Peroutka, 1987). In the same vein LY53857 is quite potent at 5-HT_2 receptors (Cohen et. al., 1983) and yet is ineffective in antagonizing the suppression of FEA by NS.

Table 11. The effect of various 5-HT antagonists on the suppression of FEA by NS.

Antagonist (μmol/kg)	Duration of Action (min) X	s.d.	Change in FEA (% of control) X	s.d.	Effect on Suppression +	-
Ketanserin (0.3-1.4)	76.5	27.8	101.2	5.7	4	0
Mianserin (1.9-18.9)	124.3	61.2	91.2	9.8	4	2
Ritanserin (0.2-2)	75	24	81.1	18.7	3	1
LY 53857 (1.3-13.1)	-	-	105.9	8.8	0	4
Metergoline (0.2-9.9)	-	-	93.5	7.8	0	4
Pizotifen (3.4-33.8)	-	-	104.3	6.6	0	4
Spiperone (0.3-1.3)	37.8	22.8	100.1	6.6	4	0
Methysergide (1.4-28.3)	-	-	84.8	14.1	0	4
Mesulergine (0.3-2.6)	12.7	3.8	96.6	6.9	2	3
ICS 205 930 (0.4-3.7)	-	-	100.2	5.7	0	4

+= Antagonized suppression of FEA by NS
-= No effect on suppression of FEA by NS

Chapter 4

Discussion

4.1 The Generation of FEA by Intracortical Pressure Ejection of Penicillin (PG)

PG has been extensively used as a convulsant in experimental models of epilepsy (Walker and Johnson, 1945; Matsumato et. al., 1969; Stark et. al., 1974; Edmonds et. al., 1974; Lockton and Holmes, 1980). PG induces two general types of epileptiform activity; 1) spike and wave activity in cats following the i.p. administration of large doses of PG (Gloor, 1984) and 2) large spikes comparable to human "interictal" spikes resulting from the direct application of high concentrations of PG to the neocortex (Ayala et. al., 1970).

In the present study the intracortical application of PG by pressure ejection (35-137 kPa) produced FEA. FEA may represent a larval form of focal penicillin spike activity (FPSA). With increasing amounts of PG, FPSA develops as the result of synchronized activity in thousands of other similarly affected neurons in the population (Walsh, 1971).

The cellular mechanism by which NS suppresses FEA is not known. However, as intracortical activity is desynchronized by NS, this may well account for the suppression of FEA at the population level. The recent observations of Vanderwolf (1987) that 5-HT contributes to

desynchronization is consistent with such an interpretation and furthermore supports the observations made in the present study.

4.2 The Site of Action For the Suppression of FEA by NS

From the systemic administration of drugs it is difficult to determine the site or sites of action at which 5-HT acts to suppress FEA. However, as the 5-HT_{1A} agonist 8-OH-DPAT, the mixed 5-HT_{1A-1B} agonists 2-MPP and TFMPP, and the blockers of 5-HT transport in fact all blocked the suppression of FEA by NS the number of possible sites at which 5-HT could act to suppress FEA is reduced. That is, depending on the sites at which the above drugs might exert their blocking action it is possible to draw some conclusions as to where 5-HT may mediate the suppression of FEA by NS.

One possible site of action for the above agents is the spinal cord where by producing analgesia they could block the suppression of FEA by NS. There is evidence, based on studies employing pharmacological, surgical, electrophysiological and dietary manipulations of central nervous system serotonergic neurotransmission suggesting that increases in the activity of brain stem (Oliveras et. al., 1974; Basbaum et. al., 1976; Belcher et. al., 1978; Willer et. al., 1979) 5-HT neurons are associated with analgesia (Messing and Lytle, 1977). Since noxious but not non-noxious stimulation results in the suppression of FEA in urethane anaesthetized rats (Neuman, 1986b), agents which

produce analgesia would be expected to block the suppression. This effect has been demonstrated for morphine (Neuman, 1986b).

The blockade of the suppression of FEA by NS following the systemic administration of the uptake inhibitors IMI and FLU, however, provides evidence that analgesia is not an important factor in the process. IMI does not appear to produce analgesia (Godefroy et. al, 1986) even at doses 3 times higher than those employed in the present study. FLU, on the other hand, has been found to produce analgesia (Messing et. al., 1976) but only at doses 10 times higher than those used in the present study.

Another possible site of action for these drugs is at the cortical level. Clearly there are 5-HT receptors located in the cortex and it is at the cortical level that the FEA is induced by application of PG. However, with the exception of 2-MPP and TFMPP, which bind to 5-HT_{1B} sites, this possibility is unlikely. First, by blocking 5-HT uptake, IMI and FLU would be expected to increase the extraneuronal level of 5-HT cortically. However, when animals are treated with reserpine or PCPA so as to reduce the level of 5-HT (Neuman, 1986a), NS is no longer effective in evoking the suppression of FEA. Thus, IMI or FLU acting solely at the cortical level might be expected to enhance the suppression of FEA and not block it.

Second, stress increases the level of 5-HT metabolites at the cortical level presumably as a consequence

of increasing the release of 5-HT (Baumann and Waldmeier, 1981). Since the source of cortical 5-HT is the dorsal raphe nucleus (O'Hearn and Molliver, 1984) and this appears to be a rather diffuse system, it is therefore likely that this source of 5-HT contributes to the normal activation of 5-HT_{1A} receptors. If these receptors are activated during stress, including NS, then it is difficult to imagine how activation of these receptors by 8-OH-DPAT, for example, could block the suppression of FEA when these same receptors would be activated by the cortical release of 5-HT.

Those agonists with 5-HT_{1B} binding affinity, 2-MPP and TFMPP, could very well act at the cortical level to block the suppression of FEA. In this case, by activating the terminal autoreceptors, 5-HT_{1B} receptors (Engel et. al., 1986), these agents could reduce the release of 5-HT.

The most likely site of action for the blockers of amine uptake and the 5-HT_{1A} agonists to block the suppression of FEA is the rostral raphe nuclei. 8-OH-DPAT and IMI by their direct and indirect action respectively can inhibit dorsal raphe neuron unit activity. Functionally the rostral raphe nuclei appear to possess inhibitory autoreceptors which is not the case for the caudal raphe nuclei (Haigler and Aghajanian, 1977). This has been confirmed by the binding site data in which very few 5-HT_{1A} receptors have been found in the midbrain raphe system with the exception of the rostral raphe where high concentrations of such binding sites are located (Pazos and Palacios, 1985).

Administering 5-HT agonists with an affinity for the 5-HT_{1A} receptor or activating these receptors by increasing the extraneuronal concentration of 5-HT in the case of IMI or FLU would thus effectively reduce the cortical concentration of 5-HT released and thereby block the suppression of FEA by NS. Thus, only at the rostral raphe does it appear that the effects of the agonists and blockers of 5-HT uptake are consistent with the results obtained with reserpine and PCPA. The observation that QPZ can antagonize the block of the suppression of FEA induced by 8-OH-DPAT and IMI is also consistent with this view. It is likely that QPZ is acting as an agonist at the cortical level, thereby mimicking the action of 5-HT, hence, restoring the suppression of FEA by NS. This interpretation is supported by the fact that QPZ has previously been found to act as an agonist at postsynaptic 5-HT receptors (Jacoby et. al., 1976; Neuman and White, 1982; Goodwin and Green, 1985).

The importance of the dorsal raphe as the site at which 8-OH-DPAT operates to block the suppression of FEA is further supported by the observations that 5-HT or 8-OH-DPAT applied locally to this region block the suppression of FEA. Although 5-HT and 8-OH-DPAT pressure ejected into the dorsal raphe region were found to block the suppression of FEA by NS, the micropipette had to contain a high concentration of drug (100 mM) before the suppression reliably occured. It is likely that a large amount of the ejected 5-HT is removed from the site both by metabolism and uptake prodesses. 5-HT has a

higher affinity for the 5-HT_{1A} receptor than 8-OH-DPAT (Hamon et. al., 1986). Therefore, even though 5-HT is taken up by an amine transporter or metabolized it can exert an effect at the same pipette concentration as 8-OH-DPAT.

There are several explanations that may account for the lengthy delay between drug ejection and the blockade of the suppression of FEA by NS. The raphe is a relatively large structure containing one of the highest concentrations of serotonergic perikarya in the brain (Palkovits et. al., 1974). It consists of three portions 1) ventral: cells between, within and just dorsal to the medial longitudinal fasciculus 2) dorsal: a small midline cluster of cells just ventral to the aqueduct of Sylvius and 3) lateral wings: a symmetrical pair of cell clusters lying laterally and dorsally in the periaqueductal gray at the level of the trochlear nucleus (O'Hearn and Molliver, 1984). It is possible that a large number of 5-HT neurons must be blocked in order to effectively suppress FEA. The application of GLU is consistent with this interpretation. The rapid reversal of the blockade following pressure ejection of GLU suggests that activity in only a small number of raphe neurons is required to restore the suppression of FEA by NS. Thus, a larger number of serotonergic neurons may have to be inhibited to block the suppression, whereas to restore the response only a few raphe neurons are required. Although consistent with the present observations this remains as speculation in the absence of direct measurement of raphe

unit activity.

Despite the long delay between drug ejection and blockade of the suppression of FEA by NS, 5-HT and 8-OH-DPAT appear to act in the vicinity of the dorsal raphe region and not at a remote site such as the median raphe, and other brainstem or spinal cord sites. The observation that the majority of sites responsive to 5-HT and 8-OH-DPAT were in the dorsal raphe while non-responsive sites were located outside the dorsal raphe suggests that the drugs are acting at a discrete site in the dorsal raphe region and not diffusing into the aqueduct to act at a site on the spinal cord. Also, the higher the concentration of 5-HT or 8-OH-DPAT pressure ejected, the shorter the delay between the pressure ejection of drug and the blockade of the suppression of FEA by NS. After finding a site at which 5-HT or 8-OH-DPAT blocked the suppression of FEA by NS, it was often possible to move the pipette by as little as $\pm$ 0.5 mm and fail to get a blockade of the suppression. This, again, suggests that the ejected drug is acting in the dorsal raphe region and not simply diffusing up the electrode track. Furthermore, responsive sites at depths closest to the aqueduct do not block the suppression of FEA with the shortest time delays.

The results obtained from the pressure ejection of 5-HT and 8-OH-DPAT into the raphe suggest that raphe activity is important in the suppression of FEA by NS. Whether raphe firing must increase following NS or whether a basal level of firing is necessary cannot be determined from the present

data, since raphe firing rates were not recorded. Further studies are required to determine the actual relationship between raphe unit activity, NS and the blockade of FEA.

Since dorsal raphe neurons project cortically (O'Hearn and Molliver, 1984), the main working hypothesis of this thesis (an increased release of 5-HT at the cortical level mediates the suppression of FEA by NS) seems plausible. Experiments conducted at the cortical level were suggestive but not unequivocal on this point.

In order to assess a possible cortical site of action for 5-HT in the suppression of FEA, two different experimental paradigms were employed: 1) 5-HTP was ejected cortically in an effort to restore the suppression in PCPA treated rats; 2) 5-HT, PCPA and FEN were applied locally to cortex in intact rats in an attempt to mimick 5-HT release. Neither experiment provided unequivocal results.

The major difficulty with these studies was most likely trying to evoke a change by applying only a small amount of drug in a localized area around the micropipette. If 5-HT is acting at the cortical level it is possible that a relatively large area of cortex needs to be affected to produce the suppression.

5-HTP pressure ejected into the cortex of PCPA treated rats only restored the suppression of FEA by NS in 2 of 4 animals. Since 5-HTP was pressure ejected unilaterally into the cortex which was bilaterally depleted of 5-HT by PCPA, it is possible that the 5-HTP could not be taken up by a

large enough number of 5-HT depleted neurons, and converted to 5-HT allowing the restoration of the suppression of FEA by NS. There are 4 cell types in the rat brain which are thought to contain aromatic L-amino acid decarboxylase, the enzyme which converts 5-HTP to 5-HT (Lovenberg, 1962). These include the pericytes lining the capillaries in the brain as well as dopaminergic, noradrenergic and serotonergic neurons (Yunger and Havey, 1976). Since immunohistochemistry reveals many capillaries which stain positive for 5-HTLI, this suggests that capillaries are taking up 5-HTP and converting it to 5-HT. Since capillaries would not release 5-HT in response to NS, the suppression of FEA by NS would not be restored if the majority of pressure ejected 5-HTP was taken up non-specifically.

The failure of 5-HT, applied cortically in untreated rats, to simulate the effect of NS at first appears inconsistent with a cortical site of action for 5-HT in suppressing FEA. As with the 5-HTP data, however, it is likely that insufficient quantities of 5-HT were present to effect a suppression of FEA. Since projections from the dorsal raphe to the cortex are bilateral (O'Hearn and Molliver, 1984), it is possible that the failure of 5-HT to simulate NS by decreasing the background level of FEA reflects the inability of unilaterally pressure ejected 5-HT to bilaterally exert an action on cortical 5-HT receptors and cause the expected suppression.

It is difficult to explain why intracortically

pressure ejected PCA simulated NS in untreated rats, while FEN had no effect on FEA, since both PCA and FEN are drugs which cause the release of 5-HT from neurons (Neckers, Bertilsson and Costa, 1976). In the cortex, PCA causes the release of 5-HT from serotonergic neurons and reduces the level of tryptophan hydroxylase to a slightly greater extent than FEN (Neckers, Bertilsson and Costa, 1976). This may possibly partially account for the ability of 10 mM PCA to suppress the background level of FEA even though 10 mM FEN failed to have the same effect. It does not explain the failure of 20 mM and 100 mM FEN to decrease the background level of FEA.

This raises the possibility that the success of PCA could be due to the non-specific effects of the drug. The immediate effects of PCA are relatively non-specific involving changes in serotonergic, noradrenergic and dopaminergic levels, while the persistent long term effects of PCA are specific to 5-HT (Sanders-Bush and Streranka, 1978). Although the ability of PCA to release NE is much lower than its ability to release 5-HT, it is possible that the reduction in the background level of FEA could have been mediated by NE. Further experiments could be performed testing PCA in PCPA treated animals to determine if the ability of PCA to suppress FEA continues following depletion of 5-HT. This would help to assess whether the action of PCA was non-specific.

GABA and 5-HT have recently been found to coexist in

the same neurons in both the dorsal raphe (Harandi et. al., 1987) and the ventral medulla oblongata (Millhorn et. al., 1987). The coexistence of the two neurotransmitters in the same neuron suggests that GABA may play an important role in modulating the release of 5-HT. Baclofen, a $GABA_B$ agonist, reduces the cortical release of 5-HT (Schlicker et. al., 1984; Gray et. al., 1986). The fact that systemically administered baclofen blocks the suppression of FEA by NS is consistent with an involvement of 5-HT in the suppression of FEA by NS. It suggests that an increased cortical concentration of 5-HT is necessary for the suppression of FEA by NS. However, it does not preclude other mechanisms by which baclofen may act. Baclofen also decreases the release of NE (Bowery et. al., 1980) which could also account for the suppression of FEA.

It can be concluded that based on data obtained in the present study, dorsal raphe involvement in the suppression of FEA by NS is certain. However, the designation of the cortex as the site of action of 5-HT requires different paradigms than those used in the present investigation.

4.3 Determination of the 5-HT Receptor Subtype Involved in Mediating the Suppression of FEA by NS

It is difficult, if not impossible, to compare drug concentrations used in binding studies with *in vivo* studies. Binding studies are conducted *in vitro* and therefore are not susceptible to problems of distribution which occur *in vivo*.

In vivo, it is not possible to determine the actual concentration of drug reaching the receptor site or sites (Peroutka, 1984). *In vitro* binding studies are often conducted in the absence of normal physiological salt solutions, which may result in an altered binding affinity compared to *in vivo* functional studies.

In *in vivo* studies, the lack of selective 5-HT antagonists for specific 5-HT receptors presents severe problems of interpretation. The 5-HT antagonists most certainly act simultaneously at multiple sites. Most 5-HT antagonists have at least weak affinity for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{2} binding sites (Leysen et. al., 1982; Leysen et. al., 1985; Conn et. al., 1986; Engel et. al., 1986). The interaction at all these sites may be very complex and with each antagonist having a different spectrum of affinities it is difficult, if not impossible, to draw firm conclusions as to the binding site at which the antagonists are acting.

Since agonists with affinity for 5-HT_{1A} and 5-HT_{1B} binding sites blocked the suppression of FEA by NS, and did not enhance it, this suggests that 5-HT_{1A} and 5-HT_{1B} binding sites did not mediate the suppression of FEA by NS. The failure of metergoline and methysergide, putative antagonists at 5-HT_{1D} binding sites (Heuring and Peroutka, 1987), to antagonize the suppression of FEA by NS suggests that 5-HT_{1D} binding sites were not involved in the process.

5-HT_{3} binding sites are also not involved in the process since ICS 205 930, a highly selective antagonist at

peripheral 5-HT_3 binding sites with very low affinity for 5-HT_1 and 5-HT_2 binding sites (Donatsch et. al., 1984), did not block the suppression of FEA by NS.

There is strong evidence that the suppression of FEA by NS is mediated by 5-HT_2 and/or 5-HT_{1C} receptors. The 5-HT_2 antagonists ketanserin, mianserin and ritanserin all blocked the suppression of FEA by NS. These 5-HT_2 antagonists also possess affinity for 5-HT_{1C} binding sites (Leysen et. al., 1982; Leysen et. al., 1985; Conn et. al., 1986; Engel et. al., 1986). The ability of mesulergine, an antagonist at both 5-HT_{1C} (Pazos et al., 1985) and 5-HT_2 binding sites (Closse, 1983), as well as the non-selective 5-HT antagonist spiperone, with affinity for 5-HT_{1A}, 5-HT_2 and 5-HT_{1C} binding sites (Richardson and Engel, 1986) to block the suppression of FEA by NS provides further evidence that 5-HT_2 and/or 5-HT_{1C} receptors mediate the process.

Both 5-HT_2 and 5-HT_{1C} receptors utilize the hydrolysis of phosphatidylinositol (PI) as an effector system (Conn et. al., 1986). In another study the systemic administration of lithium (10 mmol/kg) was found to block the suppression of FEA by NS (Neuman and Thompson, unpublished observations). In the rat brain 5-HT stimulates PI hydrolysis (Berridge et. al., 1982; Conn and Sanders-Bush, 1984; Kendall and Nahorski, 1985; Conn et. al., 1986). Lithium, by blocking myo-inositol-1-phosphatase, prevents the normal turnover of substrate for the effector system and may lower the neuronal response to agonists which stimulate

PI hydrolysis (Berridge et. al., 1982). This action may well explain the failure of NS to suppress following lithium administration. However, agonists with affinity for norepinephrine and dopamine receptors also utilize the hydrolysis of PI as a receptor mediated effector mechanism (Berridge, 1984). In the forebrain 5-HT stimulated PI turnover is not secondary to the release of any other neurotransmitter and is mediated by both 5-HT_2 and 5-HT_{1C} receptors (Conn, 1986).

The results obtained with several antagonists do not appear consistent with an action of 5-HT_2 and/or 5-HT_{1C} receptors in mediating the suppression of FEA by NS. It is difficult to explain why LY 53857, a selective and potent antagonist at 5-HT_2 binding sites (Cohen et. al., 1983), failed to antagonize the suppression of FEA by NS. Both metergoline and methysergide, antagonists with affinity for both 5-HT_2 and 5-HT_{1C} binding sites, cause a decrease in the background level of FEA, rather than a blockade of the suppression of FEA by NS. The failure of methysergide to block the suppression of FEA by NS may be explained by methysergide's action as an antagonist, a partial agonist and an agonist (Bradley et. al., 1986). Metergoline has also been reported to act as a 5-HT agonist on neurons located in the lateral geniculate nucleus, optic tectum and amygdala (Haigler and Aghajanian, 1974) as well as in hippocampal and cortical neurons (Segal, 1976; Sharma, 1977).

Based on the available data, it appears that 5-HT_2

and/or 5-HT_{1C} receptors mediate the suppression of FEA by NS. The fact that several antagonists provided inconsistent results may not be surprising, in light of the lack of correlation between electrophysiological and radioligand binding studies. The development of more specific antagonists for 5-HT_2 and 5-HT_{1C} binding sites would be required to further characterize the receptor type involved in the process using electrophysiological studies.

4.4 Evidence for 5-HT Mediating the Suppression of FEA by NS

Both systemic and local application of drugs point to a major 5-HT containing nucleus, the dorsal raphe, as a site important in mediating the suppression of FEA by NS. Systemic application of drugs known to effect 5-HT neurotransmission, including baclofen, QPZ and lithium all modify the suppression of FEA induced by NS. Finally, a number of 5-HT antagonists with actions at 5-HT_2 and 5-HT_{1C} receptors can antagonize the suppression of FEA by NS. Taken together these provide substantial evidence supporting a role for 5-HT in mediating the suppression of FEA by NS.

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