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MECHANISMS OF ACTION OF ANTIDEPRESSANT DRUGS: PRESYNAPTIC AND POSTRECEPTOR MECHANISMS

by

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List of Presentations and Publications

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I. INTRODUCTION

Clinical depression is a mood disorder and one of the most common mental illnesses. The disorder is characterized by feelings of intense sadness, and may include mental slowing, loss of concentration, anxiety, guilt feelings and pessimism. Physical symptoms manifested include insomnia or hypersomnia, anorexia, decreased energy and decreased libido. If not treated, depression can be a debilitating condition which leads with alarming frequency to suicide. The incidence of depresssion is estimated to be 5 to 10 % of the population at any one time, and thus the health cost of the disorder to society is high in terms of both treatment and job loss.

The etiology of depression is unknown. However, the most prevalent hypothesis is the "monoamine hypothesis" (Coppen, 1967), which is that depression arises from the dysregulation of monoamine neurotransmission. The notion of an alteration in transmission of brain monoamines such as 5-hydroxytryptamine (5-HT) and noradrenaline (NA) is based on several findings. Firstly, lower than normal levels of cerebrospinal fluid 5-hydroxyindole acetic acid (5-HIAA), the main metabolite of serotonin, have been found in both depressed patients with violent suicidal ideation (Asberg et al, 1976) and agressive patients (Goodwin, 1986), suggesting a link between at least agressivity, if not also depression, and dysfunctional serotonergic transmission. Secondly, iproniazid, a drug used in the treatment of tuberculosis, was serendipitously observed to have an antidepressant effect on patients who were being treated with it (Kline, 1958; Crane, 1959); this drug is known as a monoamine oxidase inhibitor and inhibits the catalysis of monoamines such as noradrenaline and serotonin. Thirdly, another study reported that the response to the 5-HT precursor L-tryptophan as measured by prolactin secretion was

decreased in depressed patients (Heninger et al, 1984). Finally, support is provided by neuroanatomy itself - serotonergic neurons project to those areas of the brain which are involved in appetite control (Saller and Stricker, 1976), sleep functions (Jouvet, 1969), attention (Davis et al, 1974) and pain perception (Lints and Harvey, 1969), all areas which could conceivably be involved in the depressive state.

Classes of Antidepressants

Drug therapy is still the main and most often used means of combatting depression. There are four groups of antidepressant drugs in common use: the tricyclic antidepressants, the "second generation" bicyclic and tetracyclic antidepressants, the monoamine oxidase inhibitors and the direct 5-HT receptor agonists. This study will focus on tricyclic and bicyclic antidepressant drugs, but a brief review of the other two classes of antidepressant drugs is warranted. A widely-used group of antidepressant drugs is the class of monoamine oxidase inhibitors. Drugs such as tranylcypramine and pargyline belong to this class and act by inhibiting the catalysis by monoamine oxidases of serotonin and NA to their metabolites in both the presynaptic neuron and the synapse. Therefore, more serotonin and noradrenaline is available for neurotransmission. A more recent set of antidepressant drugs is the direct serotonin receptor agonists. Such drugs, eg. buspirone and gepirone, are thought to act directly on 5-HT_{1A} receptors, likely at the postsynaptic site of the synapse, and thus to directly stimulate serotonergic neurotransmission in the manner of direct agonists (Gelbach and VanderMaelen, 1985; VanderMaelen et al, 1986).

Tricyclic Antidepressants and the Amine Uptake Inhibition Hypothesis

The original hypothesis as to the mechanism of action of tricyclic antidepressant drugs such as imipramine was the amine uptake blockage hypothesis. It was found that these drugs blocked the neuronal uptake of NA and 5-HT uptake at the presynaptic terminal by binding to the amine transporters, which function to bind and translocate amine molecules across the cell membrane to the inside of the cell. [^{3}H]Imipramine binds to both a high-affinity and a low-affinity site in the brain (Hrdina, 1984; Moret and Briley, 1986). The high-affinity binding of [^{3}H]imipramine to its binding site on the neuronal 5-HT transporter has been shown to be a sodium-dependent process in rat cortex (Briley and Langer, 1981; Wood, 1987) although the number of sodium ions required for the binding of one [^{3}H]imipramine molecule has been controversial. Both 1 to 1 (Wood, 1987) and 1 to 2 (Talvenheimo et al, 1983) ratios of imipramine molecules to sodium ions have been proposed based on different models of kinetic evidence. Imipramine is thought to bind to a site on the transporter adjacent to the substrate recognition site whose function it would allosterically regulate (Langer et al, 1980; Langer and Raisman, 1983).

Because the tricyclic antidepressant drugs block the uptake of NA and 5-HT at the presynaptic terminal, and because it had been hypothesized that clinical depression was largely related to impaired transmission of NA and serotonin in the brain, it followed that these drugs ultimately increased the amount of available NA and serotonin in the synapse. The extra amine would be available to act on postsynaptic receptors to increase the efficiency of the synapse.

Adaptive Neuronal Changes After Antidepressant Treatment

In the past decade it has become apparent that the blockage of 5-HT and/or noradrenaline uptake by tricyclic antidepressant drugs and selective 5-HT uptake blockers cannot be the sole mechanism by which these drugs exert their clinical effects, for two reasons. Firstly, the blockage of amine uptake would theoretically induce desensitization of postsynaptic receptors, in which case the neurotransmission of serotonin would not ultimately be increased. Secondly, these drugs act quickly (within minutes) in their amine uptake-blocking ability, and yet the alleviation of depressive symptoms does not occur for several weeks following the onset of therapy. This time delay between the onset of drug therapy and the onset of therapeutic effect is almost universal and provides the main support for the hypothesis that a neuronal adaptation in monoaminergic neurons must take place over those weeks, the end result of which is an increased neurotransmission. Research has thus focused on elucidating changes in serotonin and noradrenaline receptors following chronic antidepressant treatment.

Most antidepressant drugs ultimately affect the serotonergic transmission. There are distinct subtypes of serotonin receptors in the brain (reviewed by Peroutka and Schmidt, 1991). The 5-HT_1 subtype can be broken down into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} and has not been found to be changed by antidepressant treatment. The 5-HT_{1A} receptor has high (nM) affinity for 5-HT (Peroutka and Snyder, 1979) and is present in the highest amount in the hippocampus. The 5-HT_{1B} receptor is the putative presynaptic 5-HT autoreceptor. The 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptor subtypes are coupled to the

inhibition of adenylate cyclase. The 5-HT_{1C} receptor is present in high amount in the choroid plexus, but is scarce in other brain areas; it is coupled to the same second messenger cascade (phosphoinositide pathway) as is the 5-HT_2 receptor and may actually represent a subtype of that receptor (Hartig, 1989). Some of the functional correlates of specific serotonin receptor subtype activation are: lower lip retraction (5-HT_{1A}, Berendsen et al, 1989), penile erections (5-HT_{1C}, Berendsen et al, 1990), and head twitches (5-HT_2, Peroutka et al, 1981). The most studied serotonin receptor in depression research is the postsynaptic 5-HT_2 receptor. [^{3}H]5-HT itself binds non-selectively and with low (μM) affinity to the 5-HT_2 receptor, and thus the receptor is better labelled with the antagonist [^{3}H]ketanserin, which binds to the receptor with high (nM) affinity and greater selectivity.

A number of experiments have looked at the effects of chronic antidepressant treatment on the number of amine receptors in the brain. Banarjee et al (1977) and Peroutka and Snyder (1980) reported that chronic but not acute treatment with some antidepressants induces downregulation of ß-adrenoceptors and 5-HT_2 receptors, respectively. The tricyclic drugs imipramine and desipramine as well as amitripyline (Stockmeier and Kellar, 1986) have all been found to cause a significant downregulation of these receptors. It was hypothesized that this downregulation might in part account for the alleviation of depressive symptoms, as the downregulation was observed to correlate in timing with the therapeutic effect. In agreement with this hypothesis, it has been reported that the numbers of 5-HT_2 receptors in the post-mortem cortices of untreated suicide victims were significantly upregulated in contrast to the numbers of receptors in post-mortem cortices

of treated depressed patients who died of natural causes (Stanley and Mann, 1983; Mann et al, 1986, Hrdina et al, 1992). There has, however, been some controversy on this topic. A decreased number of 5-HT_2 sites was found in the prefrontal cortex of suicide victims compared to a control group (Gross-Isseroff et al, 1990). Similarly, Cheetham et al (1988) reported a 23 % decrease in the number of 5-HT_2 receptors in the post-mortem hippocampi of depressed suicide victims. It is possible that the discrepancy in findings between these studies may reflect psychiatric co-morbidity in the patients involved, in that potential co-existing psychiatric disorders may also exert effects on serotonergic transmission.

There are some problems with the 5-HT_2 receptor downregulation hypothesis. The most important contradiction within the theory is the fact that some antidepressants such as fluoxetine, which is a potent 5-HT uptake inhibitor, does not induce 5-HT_2 receptor downregulation after chronic treatment (Fuxe et al, 1983); this drug would be predicted to elicit downregulation as this is the classical phenomenon observed when a receptor is overstimulated with neurotransmitter. Conversely, desipramine, which does not possess significant 5-HT uptake blocking ability, does induce a downregulation of 5-HT_2 receptors after chronic treatment (Peroutka and Snyder, 1980). A second potential problem with the antidepressant-receptor downregulation hypothesis has recently been illustrated in the case of ß-adrenoceptor downregulation which was originally reported to occur in frontal cortex following chronic antidepressant treatment (Banarjee et al, 1977). Downregulation of ß-adrenoceptors has now been observed following chronic treatment with desipramine after 5 day *in vitro* exposure of rat C6 glioma cells to desipramine, suggesting that

downregulation of postsynaptic receptors following antidepressant treatment need not necessarily be secondary to increases in synaptic transmitter, but rather may be a direct result of some postsynaptic action of the drug itself (Manji et al, 1991). In this experiment, it was found not only that the number of ß-adrenoceptors was reduced, but also that the high-affinity state of the receptor was stabilized over the low-affinity site.

Another theory regarding the ultimate effect of amine uptake blockage by tricyclic antidepressant drugs has been presented and suggests that extra amine present in the synaptic cleft might overstimulate and hence downregulate or desensitize the presynaptic serotonin autoreceptors which serve to inhibit, in a negative feedback manner, the further release of serotonin from the presynaptic terminal (Chaput et al, 1987; Blier et al, 1987). The end result would be more 5-HT molecules released per impulse. Apart from the blockage of uptake of 5-HT, long-term treatment with tricyclic antidepressants (but not fluoxetine) has been reported to sensitize postsynaptic hippocampal neurons to serotonin when the latter is applied by microiontophoresis (Blier et al, 1987). Sensitization of these neurons was measured by the amount of 5-HT required to suppress the firing activity of the ascending serotonergic pathway by 50 %.

Postreceptor Action of Antidepressants

Because of the discrepancies between the immediate uptake-blocking action of the tricyclic antidepressants and the onset of alleviation of clinical symptoms, and between uptake-blocking ability and induction of ß-adrenoceptor and 5-HT_2 receptor

downregulation, it appears that there must be additional and heretofore unknown mechanisms of action of antidepressant drugs. In recent years it has been speculated that tricyclic antidepressant drugs may somehow modify the signal transduction system of the neuron. Research on the mechanism of action of antidepressant drugs has thus centred on elucidation of adaptive changes which occur in the serotonergic system over the course of treatment. Accordingly, focus has again been placed on the 5-HT_2 receptor and its associated intracellular second messenger system. A brief review of the second messenger cascade associated with this receptor is warranted.

Intracellular Pathway Linked to 5-HT_2 Receptor

The brain 5-HT_2 receptor has repeatedly been shown to be linked to the phosphoinositide pathway (Conn and Sanders-Bush, 1984, 1987; Claustre et al, 1988; Godfrey et al, 1988). Claustre et al (1988) reported an increase in cortical and hippocampal [^{3}H]inositol phosphate ([^{3}H]IP) formation following stimulation with 5-HT_2 agonists which could be blocked by ketanserin. Platelets also contain serotonin receptors and thus have been used as a tool for studying serotonergic transmission and the signal transduction systems which are linked to serotonin receptors. De Chaffoy de Corcelles et al (1988) reported that serotonin-induced phospholipid metabolism in platelets is also linked to 5-HT_2 receptor stimulation.

When the 5-HT_2 receptor is stimulated with agonist, phospholipase C (PLC) is stimulated indirectly through a G-protein. This enzyme catalyses the breakdown of membrane-bound

phosphatidyl-inositol-4,5-bis-phosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of intracellular calcium stores from storage vesicles through direct action on receptors linked to calcium channels (Berridge and Irvine, 1989). DAG binds to and stimulates protein kinase C (PKC) in the presence of phospholipid (principally phosphatidylserine) and calcium ions, perhaps by lowering its requirement for calcium. PKC proceeds to phosphorylate serine and threonine residues on a range of intracellular proteins, resulting in their activation. For example, in brain tissue, growth-associated protein-43 (GAP-43), myelin basic protein, and tyrosine hydroxylase, amongst others, are all substrates for activation by PKC (Walaas and Greengard, 1991). PKC is ultimately involved in numerous cellular functions such as secretion, exocytosis, ion channel regulation, gene expression and cell proliferation (Nishizuka, 1988). Translocation of PKC to the membrane appears to be essential in its activation. This translocation can be induced in cortical tissue by *in vitro* exposure to 5-HT_2 but not 5-HT_{1A} agonists, is inhibited by 5-HT_2 antagonists, and is dependent on extracellular calcium (Wang and Friedman, 1990).

Several experiments have provided support for the notion that translocation of PKC to the membrane serves, at least in part, to homologously desensitize the 5-HT_2 receptor. In platelets, intracellular calcium is mobilized by stimulation with serotonin. This mobilization can be inhibited by ketanserin and thus is mediated by the 5-HT_2 receptor. It was found that pretreatment with 5-HT actually abolished the calcium mobilization response to subsequent exposure to 5-HT (Kagaya et al, 1990). A role for PKC in this apparent acute desensitization of the 5-HT_2 receptor was proposed based on the finding that mezerein (activator of PKC) also inhibited the calcium release response of platelets to 5-HT.

Similarly, Dillon-Carter and Chuang (1989) reported a decrease in inositol phosphate accumulation upon stimulation of cerebellar granule cells with 5-HT_2 agonists, as well as a decrease in basal PLC activity upon administration of 4ß-phorbol 12-myristate 13-acetate (PMA) (PKC activator), and proposed a model in which PKC would phosphorylate some component of the 5-HT_2 receptor system (receptor, G-protein or PLC) and hence to inhibit the further, agonist-induced propagation of the cascade. In rat aorta, phorbol dibutyrate (PKC activator) was demonstrated to desensitize 5-HT_2 receptor-mediated phosphoinositide turnover and to attenuate the aortic contraction which is induced in this tissue by 5-HT (Roth et al, 1986), suggesting again an acute negative feedback effect of PKC on the transducing system of the 5-HT_2 receptor. This PKC-mediated negative feedback regulation is not exclusive to the 5-HT_2 receptor's second message. It has likewise been shown that other receptor transducing systems are also acutely downregulated by PKC (Nishizuka, 1986). Thus cellular signals resulting in PKC activation are followed by negative feedback inhibition in order to regulate the strength of the signal as well as to allow the cell to respond to subsequent stimulation.

Due to a "positive forward action" of PKC and a "negative feedback action" of PKC, the activity of PKC is highly regulated (reviewed by Nishizuka, 1986) and response of PKC to receptor stimulation is versatile. The immediate activation of PKC following receptor stimulation which leads to the feed-forward PKC-mediated phosphorylation of cellular proteins is thought to be important in such processes as regulation of gene expression. For example, PKC activation has been shown to ultimately result in interleukin-2 receptor induction as well as in protooncogene activation (hence the tumour promoting capacities

of PKC activators). Conversely, the negative feedback action of PKC has effects on different cellular processes. In the short-term, this negative feedback would be involved in such things as immediate homologous receptor desensitization, as with the 5-HT_2 receptor. In the long term, PKC can downregulate some receptors; the receptor for epidermal growth factor is downregulated upon long-term activation of PKC, presumably via phosphorylation of the receptor.

Action of Antidepressants on Intracellular Signalling Pathways

Some studies have reported that chronic antidepressant treatment induces desensitization of 5-HT-stimulated phosphoinositide hydrolysis as well as simultaneous downregulation of 5-HT_2 sites (Kendall and Nahorski, 1985; Conn and Sanders-Bush, 1987; Godfrey et al, 1988, Sanders-Bush et al, 1989). Godfrey et al (1988) reported a 56 % decrease in 5-HT agonist-induced head twitches (correlated to 5-HT_2 receptor number) as well as a 33 % decrease in 5-HT-stimulated cortical inositol phosphate formation following 2 week treatment with desipramine. However, 5-HT_2 receptor number has not always been correlated with phosphoinositide turnover after chronic antidepressant treatment. For example, it has been shown that 28 day treatment with sertraline, a potent 5-HT uptake inhibitor, causes no downregulation of 5-HT_2 receptors, and yet causes a decrease in [^{3}H]inositol phosphate formation upon stimulation with 5-HT (Sanders-Bush et al, 1989). Similarly, dihydroxytryptamine, which is not an antidepressant drug but rather a toxin to indoleamine neurons which causes lesions to whole serotonergic tracts, also causes no change in the number of 5-HT_2 receptors but increases 5-hydroxytryptophan-induced

phosphoinositide turnover in the cortex and brainstem (Butler et al, 1990). Thus it is apparent that treatments with drugs can affect one aspect of the receptor-second message system without causing a change in the other. It is possible that antidepressant drugs, which appear to have varying effects on the number of postsynaptic 5-HT_2 receptors, may actually have in common another action, possibly inside the cell on some component of the associated second messenger system.

An interesting study has been done on the effect of chronic antidepressant treatment on the activity of protein kinase A (PKA), an enzyme which is not associated with the 5-HT_2 receptor message but is associated with other brain receptors such as the 5-HT_{1A} receptor and the ß-adrenoceptor. It was found that chronic treatment with imipramine or tranylcypramine as well as electroconvulsive shock therapy (an alternative form of treatment for depression) resulted in an increase in the amount of PKA associated with the membrane fraction of cortical cells; upon further investigation, the activity of PKA was found to be decreased in the cytosolic fraction of the cell and increased in the nuclear fraction (Nestler et al, 1989). It was postulated that the translocation of PKA to the nucleus might result in the phosphorylation of nuclear proteins which would in turn have actions at the gene level. While the results of this particular experiment are dubious because of a lack of purification of the nuclear pellet and hence probable contamination of this fraction, the study raises the possibility that intracellular enzymes which mediate the phosphorylation and activation of cellular proteins may well be involved in the mechanism of action of antidepressant drugs.

Other studies involving PKA and the associated cAMP have shown that chronic antidepressant treatment may alter the cAMP-dependent phosphorylation system of the neuron. It was reported that chronic but not acute or *in vitro* desipramine treatment increases the amount of regulatory subunits of PKA which were isolated by column chromatography from cerebrocortical soluble fractions of rat brain (Brunello et al, 1990). Binding of radiolabelled cAMP to the regulatory subunit of PKA was also increased following both chronic desipramine and fluoxetine treatment (Perez et al, 1991). Chronic desipramine treatment resulted in increased basal and cAMP-stimulated phosphorylation (incorporation of [^{32}P]ATP) of a 280 kDa cAMP-dependent phoshoprotein (Perez et al, 1989) which for its biochemical properties resembles MAP-2 (microtubule-associated protein-2), a cellular protein which regulates microtubule function in dendrites and perikarya (Theurkauf and Vallee, 1983). Further experiments revealed that, following chronic desipramine treatment, the phosphorylation of MAP-2 in cortical microtubule fraction was increased (Perez et al, 1989). It was postulated that desipramine, acting via ß- and α_2-adrenoceptors, and fluoxetine, acting via 5-HT receptors coupled to adenylate cyclase, result after long-term treatment in an alteration both in PKA activation and in PKA-mediated phosphorylation. The increased activation of MAP-2, for example, may be significant in terms of intracellular transduction of signals to the nucleus, because MAP-2 is highly concentrated in dendrites, the neuronal structures which receive synaptic input. Therefore, it appears that chronic antidepressant treatment may at least in part exert its effects through changes in protein phosphorylation which in turn would affect the control of cellular functions.

PKC has been implicated in more cellular events than simply activation of protein targets and homologous desensitization of receptors. For example, Tuominen et al (1991) proposed a nuclear role for PKC in cultured bovine adrenal medulla cells. Upon stimulation of these cells with angiotensin II agonists, PKC activity in the particulate fraction was significantly increased. A PKC activator had previously been shown to increase proenkephalin gene expression (Kley, 1988; Stachowiak et al, 1989), and angiotensin II had been found to induce the production of this neuropeptide (Stachowiak et al, 1989); it was proposed that PKC itself may induce gene expression. In the brain, an increase in membrane-bound PKC activity was reported in the hippocampus following hippocampal kindling (Daigen et al, 1991); thus, activation of PKC may play a role in the kindling phenomenon. PKC has also been implicated in hippocampal noradrenaline release (Huang et al, 1988) after phorbol ester (PKC activator) was found to induce the release of this transmitter in rabbit hippocampal slices. Activation of PKC in terms of translocation of PKC activity from the cytosol to the membrane compartments of CA1 hippocampal neurons has been reported to occur following associative learning in rabbits (Olds et al, 1989); it was proposed that PKC activation may play a pivotal role in the storage and expression of memory.

There has been no attempt to date to elucidate the potential effects of chronic antidepressant treatment on PKC location or activity within serotonergic neurons. Because this enzyme's action forms a portion of the second message of the 5-HT_2 receptor, it would be important to determine any modifications of its location or activity or its response to 5-HT_2 receptor stimulation following chronic antidepressant treatment.

Aims

A. Presynaptic mechanisms: Antidepressants and serotonin transporter function

Paroxetine, a novel antidepressant drug which selectively blocks the uptake of 5-HT, binds with high affinity to a single class of sites and is thought to bind to the 5-HT recognition site itself (Habert et al, 1985; Humphreys et al, 1988; Marcusson et al, 1988; Graham et al, 1989; Hrdina et al, 1990). It has been reported that [^{3}H]paroxetine is a more selective label of serotonin uptake sites than is [^{3}H]imipramine (Hrdina et al, 1990). The kinetics of sodium dependence of [^{3}H]paroxetine binding have not yet been demonstrated, and may yield new insight into its presynaptic mechanism of action. In this regard, the kinetics of sodium dependence of [^{3}H]paroxetine binding will be evaluated in membranes and sections of rat diencephalon, an area rich in serotonergic innervation (Saavedra et al, 1977; Parent et al, 1981; Hrdina et al, 1990). The diencephalon area, particularly the midbrain raphe, has also been directly shown to contain a high number of high-affinity (pM) [^{3}H]paroxetine binding sites (de Souza and Kuyatt, 1987; Hrdina et al, 1990; Dewar et al, 1991).

The sodium dependence of [^{3}H]5-HT uptake kinetics will be assessed in synaptosomes from the same brain area. Although this dependence has been extensively studied in

other brain areas (Blackburn et al, 1967; Stauderman and Jones, 1985; Kilpatrick et al, 1986; Wood, 1987; O'Reilly and Reith, 1988) as well as in platelets (Sneddon, 1969; Rudnick, 1977), it has not been evaluated in the diencephalon; in addition, there has been considerable controversy as to the number of sodium ions which are required for the binding and then for the subsequent translocation of one 5-HT molecule. These two phases of the uptake process are distinct (Wood and Wyllie, 1982) and hence their sodium dependence can be considered separately. The kinetics of [^{3}H]5-HT uptake in the cortex and hippocampus following chronic antidepressant treatment will also be measured.

B. Postreceptor mechanisms: Antidepressants and PKC

The effect of acute and chronic treatment with antidepressants on PKC location and activity in both soluble and particulate fractions of brain tissue will be assessed. The brain areas studied will be the fronto-parietal cortex and the hippocampus as autoradiography has revealed that these areas contain the largest amounts of PKC (Worley et al, 1986; El-Fakahany et al, 1988; Horsburgh et al, 1991; Kawagoe et al, 1991). The drugs which will be used will be desipramine and fluoxetine in order to assess the effect on PKC of treatment with both a 5-HT uptake blocker which does not induce downregulation of 5-HT_2 receptors after chronic treatment (fluoxetine), and with a drug without 5-HT uptake blocking ability but which does induce a downregulation of 5-HT_2 receptors (desipramine). The effect of acute treatment with antidepressants will be assessed in order to differentiate long-term effects from immediate effects of the drug upon administration. Pandey et al

(1991[a]) found an increase in [^{3}H]PIP_2 and a decrease in [^{3}H]IP_3 in human platelets following *in vitro* exposure to desipramine and imipramine, and suggested acute inhibitory actions of tricyclic antidepressants on PLC, making apparent the need for assessment of the effects of acute antidepressant treatment on PKC.

One possibility is that, while PKC location or activity may not change after chronic antidepressant treatment, there may be a change in the response to 5-HT_2 receptor stimulation in terms of the relative distribution of PKC activity between the subcellular fractions. For example, it was reported that chronic lithium treatment had no effect on either PKC location or basal activity in either subcellular fraction of rat hippocampus; however, PKC-mediated phosphorylation of specific target proteins was altered by the treatment (Casebolt and Jope, 1991). Therefore, a separate set of experiments will be performed in which the subcellular distribution of PKC activity will be measured after acute stimulation of the receptor with the selective 5-HT_2 receptor agonist 1 - (2,5 - dimethoxy - 4 - iodophenyl) - 2 - aminopropane (DOI). DOI labels the high-affinity, guanyl nucleotide-sensitive state of the 5-HT_2 receptor as well as the 5-HT_{1C} receptor, and specific binding of DOI is highest in layer IV of the anterior cortex and low in the hippocampus (Appel et al, 1990). Berendsen and Broekkamp (1991) reported that chronic treatment with mianserin, a tetracyclic 5-HT_2 antagonist, resulted in a decrease in DOI-induced but not basal head twitches, a behavioural phenomenon associated with 5-HT_2 receptor activation; hence DOI is likely an appropriate drug to use to challenge the 5-HT_2 receptor when attempting to differentiate between basal second messenger activity and receptor-stimulated second messenger function. The effect of chronic antidepressant treatment on

the distribution of PKC following 5-HT_2 receptor stimulation with DOI will thus be evaluated.

Both the location and activity of PKC will be measured. The enzyme exists in at least 3 different isozymic forms, types I, II and III which have been found to be heterogeneously distributed in rat brain (Huang et al, 1987). Type I PKC is brain-specific and high amounts have been reported in rat cerebellum. In contrast, type II PKC is predominant in the cortex, and type III PKC in the olfactory bulb (Huang et al, 1987). The distribution of PKC within cells is also heterogenous (reviewed in Huang, 1989). Type I isozyme of PKC has been shown to be localized in cerebellar Purkinje cells to the cell body and dendrites, with very little present in nuclei and axons although it has been found in synaptosomes of the same cells. The presence of this isozyme in the dendrites and in synaptosomes suggest potential roles for it in signal transduction and transmitter release, respectively. In hippocampal granule and pyramidal cells, it has been shown that types I and II isozyme immunoreactivity is present throughout entire cell bodies, while type III isozyme immunoreactivity is present exclusively in the cytoplasm. In the present study, PKC isozymes will not be differentiated in terms of either location or activity. PKC location will be measured by the binding of [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) in cellular fractions; [^{3}H]PDBu is a phorbol ester which binds to PKC, its structure ressembling that of DAG. Phorbol esters are tumour promoters and potent activators of PKC (Kraft and Andersom, 1983; Bell and Burns, 1991). Unlike DAG, phorbol esters are capable of permeating the cell to reach and activate both cytosolic and intracellular membrane-associated PKC (Nahorski et al, 1986). PKC activity will be measured by the incorporation

of [^{32}P]ATP into a specific peptide target of PKC.

Hypotheses

A. Presynaptic mechanisms: Antidepressants and serotonin transporter function

Because [^{3}H]paroxetine selectively labels the serotonin transporter and is thought to bind to the substrate recognition site of 5-HT on the neuronal serotonin transporter, it is hypothesized that the sodium requirement for binding of [^{3}H]paroxetine to the transporter will be the same as that for binding of [^{3}H]5-HT to the transporter.

B. Postreceptor mechanisms: Antidepressants and PKC

It is hypothesized that PKC location and/or PKC basal activity will translocate from the cytosol to the membrane fraction following chronic but not acute antidepressant treatment, representing a potential translocation to the nucleus, and that 5-HT$_2$ receptor-stimulated PKC activity following chronic antidepressant treatment will be distributed between the subcellular fractions in a different ratio than that in unchallenged controls.

II. MATERIALS AND METHODS

A. PRESYNAPTIC MECHANISMS: ANTIDEPRESSANTS AND SEROTONIN TRANSPORTER FUNCTION

1. Experimental design

Male Sprague-Dawley rats weighing 150 to 175 g (or 300 to 400 g if for use in experiments involving either [^{3}H]paroxetine or [^{3}H]PDBu binding in brain sections) were obtained from Charles-River Inc., and were used for all experiments. Animals were housed under standard laboratory conditions (12 hour light/dark cycle) with access to food and water *ad libitum*. To assess their sodium dependence, [^{3}H]paroxetine binding and [^{3}H]5-HT uptake were each measured in the presence of different concentrations of NaCl in the respective incubation buffers. The concentrations of NaCl used were 0, 15, 30, 60, 120 and 200 mM ([^{3}H]paroxetine binding in homogenates), 0, 15, 30, 60, 120, 200 and 300 mM ([^{3}H]paroxetine binding in brain sections) or 0, 15, 30, 60 and 120 mM ([^{3}H]5-HT uptake).

2. Experimental procedures

2.1 [^{3}H]Paroxetine binding in homogenates

[^{3}H]Paroxetine binding in homogenates was determined as described by Habert et al (1985).

2.1.1 *Preparation of tissue*

Rats were killed by decapitation, their brains dissected out and quickly removed. A coronal segment of diencephalon (from 3 to 7 mm anterior to the interaural line) was homogenized in 200 volumes of incubation buffer (50 mM Tris-HCl, 5 mM KCl and concentrations of NaCl ranging from 0 to 200 mM, pH 7.4) for 15 seconds with a Polytron (setting 6). The following concentrations of NaCl were used: 0, 15, 30, 60, 120 and 200 mM. The effect of NaCl on [^{3}H]paroxetine binding was assessed without equiosmotic replacement of Na^+ in the incubation buffer; preliminary experiments showed that [^{3}H]paroxetine binding at lower NaCl concentrations (below 120 mM) without Na^+ replacement was not significantly different from that seen in a buffer with Na^+ equiosmotically replaced by Tris-HCl.

The homogenate was centrifuged at 30,000g and 4^{0}C for 10 minutes; the resulting pellet was resuspended in the original volume of buffer, homogenized and centrifuged again under the identical conditions. The final pellet obtained was resuspended in the original volume of buffer and kept on ice.

2.1.2 *Binding assay*

Assay tubes (with final incubation volumes of 1.6 mL) contained 550 μL of incubation buffer (in the absence or presence of 10 μM fluoxetine, for determination of total and non-specific binding, respectively) and 50 μL of [^{3}H]paroxetine at concentrations of 0.05, 0.1,

0.25, 0.5, 1.0 or 2.0 nM. Incubation was started by the addition to each tube of 1000 μL homogenate, corresponding to final protein concentrations per tube of 350 to 400 μg. Incubation was performed in triplicate and proceeded for one hour at room temperature (20 to 22^{0}C). Termination was achieved by the addition of 5 mL of ice-cold incubation buffer, subsequent filtration through Whatman GF/B filter discs (dipped in ice-cold inbubation buffer containing 0.1 % polyethyleneimine), and two additional washes. The filters were dried and the retained radioactivity was determined by liquid scintillation spectrometry (Beckman LS 7800) at a counting efficiency of 47 %. The fraction of specific bound dpm to total dpm added ranged between 4 and 10 % and that of total bound dpm to total dpm added between 8 and 16 % depending on the concentration of the ligand. The specific binding of [^{3}H]paroxetine at 0.25 nM [^{3}H]paroxetine (chosen to approximate the Kd value of binding) amounted to between 60 and 90 % of total binding.

2.1.3 *Determination of binding parameters*

The maximum number of binding sites (Bmax) and equilibrium dissociation constant (Kd) were calculated by Scatchard analysis of saturation binding data.

2.2 [^{3}H]Paroxetine binding in brain sections

[^{3}H]Paroxetine binding in sections was determined as described by Hrdina et al (1990).

2.2.1 *Preparation of tissue*

Rats weighing 300 to 400 g were anaesthetized with an intraperitoneal injection of sodium pentobarbital and perfused via cannulated left heart ventricle with 60 mL of Krebs-phosphate buffer (pH 7.3) containing 0.16 M sucrose and 0.6 mL heparin solution (2 USP units/mL), followed by 60 mL of the same buffer solution containing 0.1 % formaldehyde. Follwing perfusion and decapitation, brains were quickly removed, frozen on dry ice and stored at -80°C for 24 hours. Brains were mounted on cryostat (Reichert Jung Cryocut E) chucks and coronal sections of 10 μm thickness from 4 brains 6 to 7 mm anterior to the interaural line were cut at -18°C and thaw-mounted onto gelatin-coated microscope slides. Slides were kept at -20°C for 48 hours before being used.

2.2.2 *Binding assay*

To determine [^{3}H]paroxetine binding, sections were warmed to room temperature and preincubated in Coplin jars containing 30 mL of 50 mM Tris-HCl buffer (pH 7.4) for 15 minutes at room temperature (20 to 22°C). Sections were transferred to jars containing 30 mL of the same buffer containing 0.4 nM [^{3}H]paroxetine (chosen to approximate the Kd value of binding) and the following concentrations of NaCl: 0, 15, 30, 60, 120, 200 or 300 mM. Incubation proceeded in duplicate for one hour at room temperature (temperature selected to give an optimum specific/non-specific signal ratio). To estimate non-specific binding, adjacent sections were incubated in the presence of 30 μM fluoxetine. Incubation was terminated by the washing of sections twice in incubation buffer

at 37°C for 20 minutes, rinsing in distilled water, and wiping off of slides with Whatman GF/B filter discs for liquid scintillation counting. The specific binding of [^{3}H]paroxetine amounted to between 60 and 90 % of total binding.

2.3 [^{3}H]5-HT uptake

[^{3}H]5-HT uptake was measured as described by Mann and Hrdina (1992).

2.3.1 *Preparation of tissue*

Rats were killed by decapitation and their brains dissected out and quickly removed. A coronal segment of diencephalon (from 3 to 7 mm anterior to the interaural line) was homogenized in 10 volumes of sucrose buffer (0.32 M sucrose, 0.002 M Tris-HCl, pH 7.4) with a Teflon pestle homogenizer. The homogenate was centrifuged at 100g and 4°C for 10 minutes and the resulting supernatant centrifuged at 17,000g and 4°C for 20 minutes. The final pellet, representing the synaptosomal (P_2) fraction, was resuspended in the original volume of buffer and kept on ice.

2.3.2 *Uptake assay*

Assay tubes (with final incubation volumes of 0.5 mL) contained 400 μL of incubation buffer (20 mM Tris-HCl, 5 mM KCl, 1.2 mM $MgCl_2.6H_2O$, 2.5 mM $CaCl_2.2H_2O$, 10 mM glucose, 0,1 mM pargyline, 1 mM ascorbic acid and concentrations of NaCl ranging from

0 to 120 mM, pH 7.4) aerated with 95 % O_2 and 5 % CO_2. The following NaCl concentrations were used: 0, 15, 30, 60 or 120 mM. Preliminary experiments showed that [^{3}H]5-HT uptake at lower NaCl concentrations (below 120 mM) without Na^+ replacement was significantly reduced in comparison to uptake measured in an incubation buffer with Na^+ equiosmotically replaced by LiCl. It has been demonstrated earlier (Kilpatrick et al, 1986) that lithium possessed no intrinsic activity towards the uptake pump for 5-HT indicating the suitability of this ion for replacement of sodium in serotonin uptake assays (Wood, 1987; O'Reilly and Reith, 1988). In contrast, a reduced [^{3}H]5-HT accumulation has been reported to occur when Na^+ was replaced by $Tris^+$ in the incubation medium (Stauderman and Jones, 1985). To assess the effect of lower sodium concentrations (below 120 mM) on serotonin uptake and maintain the osmolality of the buffer, NaCl was therefore replaced by equiosmolar amonuts of LiCl.

Fifty μL of homogenate corresponding to 200 to 250 μg protein per tube was added to tubes which were then preincubated at 37°C for 7 minutes. Fifty μL of [^{3}H]5-HT at concentrations of 40, 50, 65 or 100 nM was then added to each tube and a 2 minute incubation at 37°C in triplicate followed. Blank tubes representing non-specific uptake were incubated at 0°C. Incubation was terminated by the addition of 5 mL of ice-cold incubation buffer and subsequent filtration through Whatman GF/B filter discs (dipped in ice-cold incubation buffer) followed by 2 additional washes. The filters were dried and the retained radioactivity determined by liquid scintillation spectrometry. Net uptake was defined as the difference between the total (37°C incubation) and the non-specific (0°C incubation) uptake. Net uptake amounted to between 75 and 85 % of total binding.

2.3.3 *Determination of uptake parameters*

The maximum uptake rate (Vmax) and affinity constant (Km) were calculated from Lineweaver-Burke plots.

Note: To assess the effect of chronic antidepressant treatment on [^{3}H]5-HT uptake, measurement of [^{3}H]5-HT uptake was done as described in the Section 2.3 with the following exceptions:

i) The brain areas of the treated animals which were used in the assay were the cortex and the hippocampus.

ii) The NaCl concentration of 120 mM was maintained in the incubation buffer.

iii) The incubation proceeded for 5 minutes.

B. POSTRECEPTOR MECHANISMS: ANTIDEPRESSANTS AND PKC

1. Experimental design

Experiments were performed to evaluate the effects of chronic treatment with antidepressants on [^{3}H]5-HT uptake, [^{3}H]PDBu binding and PKC activity (both basal and DOI-challenged). Animals were injected intraperitoneally daily with desipramine (10 mg/kg), fluoxetine (5 mg/kg) or the equivalent volume of 0.9 % saline as a vehicle. The doses selected were based on the therapeutic doses used clinically (daily dose of

desipramine = 75 to 200 mg; daily dose of fluoxetine = 20 to 80 mg). Animals were treated for 21 days and sacrificed 24 hours later in the case of desipramine or 48 hours later in the case of fluoxetine. The time periods between the last daily dose of drug and sacrifice were chosen to avoid residual drug interference with the assays, and were based on the difference in plasma half-lives between the 2 drugs ($t_{1/2}$ of desipramine = 14 to 62 hours; $t_{1/2}$ of fluoxetine = 24 to 96 hours).

2. Experimental procedures

2.1 [^{3}H]PDBu binding in brain sections

[^{3}H]PDBu binding in brain sections was determined as described by Worley et al (1986).

2.1.1 *Preparation of tissue*

Rats were perfused and the diencephalon areas 5 to 6 mm anterior to the interaural line were cut into sections of 15 μm thickness as described above (see above in 2.2 "[^{3}H]Paroxetine binding in brain sections") .

2.1.2 *Binding assay*

Sections were incubated for one hour at 33°C in incubation buffer (50 mM Tris-HCl, 1 mM

$CaCl_2$ and 100 mM NaCl, pH 7.7) containing 2.5 nM [^{3}H]PDBu (chosen to approximate the Kd value of binding). To estimate non-specific binding, adjacent sections were incubated in the presence of 1 μM cold PDBu. Specific binding amounted to between 70 and 90 % of total binding. Incubation was terminated by the washing of sections twice in ice-cold incubation buffer for 2 minutes and rinsing in ice-cold distilled water. For non-autoradiographic studies, sections were then wiped from slides with Whatman GF/B filter discs and the retained radioactivity measured by liquid scintillation spectrometry. Sections destined for autoradiography were dried and apposed to tritium-sensitive Hyperfilm (Amersham, Des Plaines, IL) and kept at 4^{0}C for 4 days to generate autoradiograms. Every tenth section was stained with cresyl violet to facilitate the identification of brain structures by using the atlas of Paxinos and Watson (1986).

2.2 [^{3}H]PDBu binding in homogenates

[^{3}H]PDBu binding in homogenates was determined as described by Horsburgh et al (1991).

2.2.1 *Preparation of tissue*

Rats were killed by decapitation, their brains dissected out and quickly removed. Cortical or hippocampal tissue was homogenized in 10 volumes of homogenate buffer (0.32 M sucrose, 5 mM benzamidine, 2 mM dithiothreitol, 3 mM EGTA, 0.5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, 0.1 mM phenylmethylsulfonylfluoride, 0.1 mg/mL leupeptin, 0.05 mg/mL pepstatin

and 0.1 mg/mL aprotinin) for 10 seconds using a Polytron (setting 6). Homogenates were then centrifuged at 10,000g and 4°C for 8 minutes to precipitate nuclei and cytoskeleton. The resulting supernatant was then ultracentrifuged (Beckman L7 - 35) at 100,000g at 4°C for one hour. The resulting pellet, resuspended in the original volume of buffer, and supernatant constituted the particulate and soluble fractions of the tissue, respectively.

2.2.2 *Binding assay*

Assay tubes (with final incubation volumes of one mL) contained 930 μL of incubation buffer (50 mM Tris-HCl, 10 mM $(CH_3COO)_2Mg.4H_2O$, 1.4 mM $CaCl_2$, 0.4 mM EGTA, 50 mM KCl, 4 mg/mL BSA and 100 μg/mL phosphatidylserine, pH 7.5) in the absence or presence of 2 μM cold PDBu (for determination of total and non-specific binding, respectively) and 50 μL of 2.5 nM [^{3}H]PDBu (chosen to approximate the Kd value). Incubation was started by the addition of 20 μL homogenate, corresponding to approximately 25 to 35 μg protein in the soluble fraction and 15 to 25 μg in the particulate fraction. Incubation proceeded in triplicate at 4°C for 2 hours, and was terminated by the addition of 5 mL ice-cold filtration buffer (20 mM Tris-HCl, 1 mM $CaCl_2$ and 10 mM $(CH_3COO)_2Mg.H_2O$, pH 7.5), subsequent filtration through Whatman GF/B filter discs (dipped in ice-cold 20 mM Tris-HCl buffer containing 0.1 % polyethyleneimine) and 4 additional washes. The filters were dried and the retained radioactivity was determined by liquid scintillation spectrometry. The specific binding of [^{3}H]PDBu in the soluble fraction amounted to between 95 and 98 % of total binding and between 75 and 85 % in the particulate fraction.

2.2.3 *Competition studies - displacement of cold drug*

To assess the displacement of [^{3}H]PDBu binding by cold drug, the soluble fraction of cortical tissue (n = 4) was prepared as described in Section 2.2.1. The binding assay proceeded as described in Section 2.2.2, with the exception that assay tubes were incubated in the presence of 2.5 nM [^{3}H]PDBu and increasing concentrations of cold PDBu (0 to 20 μM). The 14 concentrations of cold PDBu used were: 0, 2 x 10^{-7}, 2 x 10^{-6}, 2 x 10^{-5}, 2 x 10^{-4}, 6 x 10^{-4}, 0.002, 0.006, 0.02, 0.06, 0.2, 0.6, 2 and 20 μM. Incubation proceeded in duplicate for 2 hours.

2.3 Measurement of protein kinase C activity

Protein kinase C activity in cellular fractions was measured using a commercially-available enzyme assay system (Amersham, Des Plaines, IL). A PKC-specific target peptide and all necessary co-factors were provided in the kit.

2.3.1 *Preparation of tissue*

Soluble and particulate fractions of cortical or hippocampal tissues were prepared as described above (see "[^{3}H]PDBu binding in homogenates"). Soluble fractions were subsequently diluted 8 times in homogenate buffer, and particulate fractions 4 times.

2.3.2 *Activity assay*

Eppendorf assay tubes (with final incubation volumes of 75 μL) contained 25 μL of component mixture (3 mM $Ca(C_2H_3O_2)_2$, 2 mole % Lαphosphatidyl-L-serine, 6μg/mL phorbol 12-myristate 13-acetate, 225 μM peptide and 7.5 mM dithiothreitol in 50 mM Tris-HCl containing 0.05 volumes sodium azide, pH 7.5) and 25 μL homogenate, corresponding to 7 to 9 μg and 6 to 8 μg protein in the soluble and particulate fractions respectively (blank tubes representing non-specific phosphorylation contained 25 μL homogenate buffer instead).

The reaction was initiated by the addition of 25 μL of Mg-ATP buffer (10 μCi/mL [^{32}P]ATP, 150 μM ATP and 45 mM $(CH_3COO)_2Mg.4H_2O$ in 50 mM Tris-HCl containing 0.05 volumes sodium azide, pH 7.5) to each tube. Incubation proceeded for 15 minutes at room temperature (25^{o}C) and was terminated by the addition of 100 μL stop reagent (dilute acidic reaction-quenching reagent) to each tube. An aliquot of solution from each tube (125 μL) was blotted onto individual peptide-binding papers which were then placed in a 5 % acetic acid bath (10 mL per paper) for 10 minutes at room temperature. This solution was then decanted and replaced with fresh 5 % acetic acid for a second 10 minute wash. Papers were dried and the retained radioactivity determined by liquid scintillation spectrometry.

2.3.3 *Calculation of PKC activity*

The number of pmoles phosphate transferred per minute by PKC to the PKC-specific peptide substrate was calculated from the following equation:

$$P = \frac{T \times 10^3}{I \times R} \text{ pmoles/minute}$$

where T = total phosphate transferred to peptide = sample cpm x 1.4 (total terminated assay volume divided by the volume applied to the binding paper) - blanks cpm

I = incubation time = 15 minutes

R = specific radioactivity of 150 μM magnesium [^{32}P]ATP = cpm per 10 μL magnesium [^{32}P]ATP/1.5 nmol (10 μL of 150 μL ATP contains 1.5 nmoles)

Determination of protein content

Protein content of assay tubes from [^{3}H]paroxetine binding assays and [^{3}H]5-HT uptake assays were determined using the method of Lowry et al (1951). A Beckman DU - 7 spectrophotometer was used to measure the absorbance of light by proteins at a wavelength of 640 nm. A revised version of this method was used to determine the

protein content of tubes from [^{3}H]PDBu binding assays and PKC activity assays in order to avoid interference in the Lowry assay by reagents present in the homogenate buffer. Aliquots of homogenate were made up to a volume of 950 μL with distilled water and 50 μL of absolute trichloroacetic acid was added to each tube to precipitate proteins. The tubes were then centifuged (Eppendorf 5412 benchtop centrifuge) for 5 minutes to pellet out proteins. The resulting supernatants were aspirated and discarded, and the resulting pellets were resuspended in 200 μL of 0.1 M NaOH. The method of Lowry et al (1951) was subsequently used to determine the protein content of each tube.

Drugs and chemicals

[^{3}H]Paroxetine (specific activity, 25 Ci/mmol), [^{3}H]5-HT creatinine sulfate (specific activity, 23.2 Ci/mmol), [^{3}H]PDBu (specific activity, 18.6 Ci/mmol) and [^{32}P]ATP tetra(triethylammonium) salt (specific activity, 3000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). PDBu was purchased from Sigma Chemical Co. (St. Louis, MO), (±)-DOI hydrochloride from Research Biochemicals Inc. (Natick, MA) and the protein kinase C enzyme assay system from Amersham (Des Plaines, IL). Fluoxetine hydrochloride was generously donated by Eli Lilly & Co. (Indianapolis, IN), pargyline hydrochloride by Sigma Chemical Co. (St. Louis, MO) and desipramine hydrochloride by Ciba-Geigy Canada Ltd. Other chemicals used were of purest grade available.

Analysis of data

Kinetic parameters (Kd and Bmax) of [^{3}H]paroxetine binding were generated by using the BDATA computer program (EMF Software, Inc., Knoxville, TN). The competition curve of [^{3}H]paroxetine binding was generated by using the CDATA computer program (EMF Software, Inc., Knoxville, TN). Linear regression lines were fitted using a weighted least squares method and sodium dependence curves were fitted to a rectangular hyperbola by a non-linear least square computer program.

Autoradiograms were quantified by computerized densitometry and image analysis using the Microcomputer Imaging Device (MCID, Imaging Research, St. Catherines, Ontario). Standards were prepared by addition of varying known amounts of radioactivity to samples of brain paste which were then frozen, cut and apposed to the Hyperfilm in the same manner and for the same period of time as corresponding brain tissue sections labelled with [^{3}H]PDBu. Binding densities were calculated from a calibration curve obtained for the brain paste standards and were expressed as femtomoles per miiligram of protein. Protein content of 15 μm brain sections was determined in randomly selected sections by the method of Lowry et al (1951) and was found to be 1.8 to 2.2 mg per section in sections from the brain area 5 mm anterior to the interaural line. This value was then used to convert radioactivity or optical density readings into fmol/mg protein. The density of non-specific binding was measured in corresponding areas in adjacent sections and subtracted from the total binding to obtain the specific binding.

Statistics

Data are expressed as mean ± S.E.M. One-way ANOVA or Student's t test were used to calculate the statistical difference between the means whenever appropriate.

III. RESULTS

A. PRESYNAPTIC MECHANISMS: ANTIDEPRESSANTS AND SEROTONIN TRANSPORTER FUNCTION

1. Sodium dependence of [^{3}H]paroxetine binding

In membrane preparations from rat diencephalon, [^{3}H]paroxetine binds to a single population of sites. Scatchard analysis of saturation binding yielded a linear plot with a mean Kd (equilibrium dissociation constant) value of 0.28 ± 0.04 nM and Bmax (maximum number of binding sites) of 500 ± 36 fmol/mg protein (n = 4) at the optimum (60 mM) NaCl concentration (Figs. 1, 2, 3, Table 1). Analysis of equilibrium binding of [^{3}H]paroxetine at various sodium concentrations in the incubation medium reported in Figs. 1 and 2 shows that [^{3}H]paroxetine binding saturates at each external sodium concentration and that virtually the same number of sites is available at each sodium concentration tested. Decrease in sodium concentration failed to alter the calculated Bmax values (Figs. 2, 3, Table 1). [^{3}H]Paroxetine binding at 0 mM NaCl was so low compared to that at all other concentrations of sodium tested that the Bmax value could not be calculated (Figs. 1,2). In contrast, the affinity (1/Kd) of [^{3}H]paroxetine for its binding sites was significantly affected by altered sodium concentration (ANOVA, $f = 3.942$, $p < 0.024$) (Fig. 3). By decreasing the sodium concentration from 60 mM to 15 mM, the Kd of [^{3}H]paroxetine binding increased from 0.28 ± 0.04 to 0.51 ± 0.02 nM (n = 4) (Table 1). The relationship of external sodium concentration to the change in affinity of [^{3}H]paroxetine binding (1/Kd) illustrated in Fig. 3 can best be described as a simple rectangular hyperbola ($r = 0.893$; $p < 0.025$) to be expected when one sodium ion is

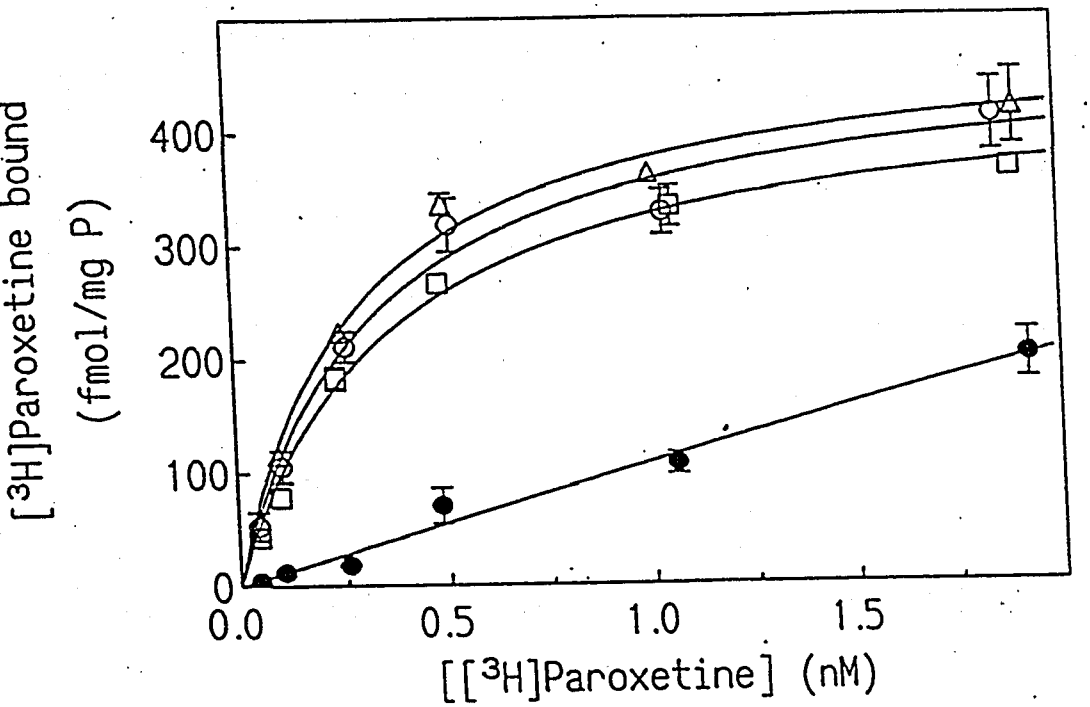


Fig. 1. Saturation curves for specific binding of [^{3}H]paroxetine in membranes from rat diencephalon at increasing concentrations of sodium: 0 (●), 15 (□), 30 (△) and 120 (○) mM NaCl. Binding was determined over a concentration range of [^{3}H]paroxetine of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 10 μM fluoxetine. Each point represents the mean ± SEM of four separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol.

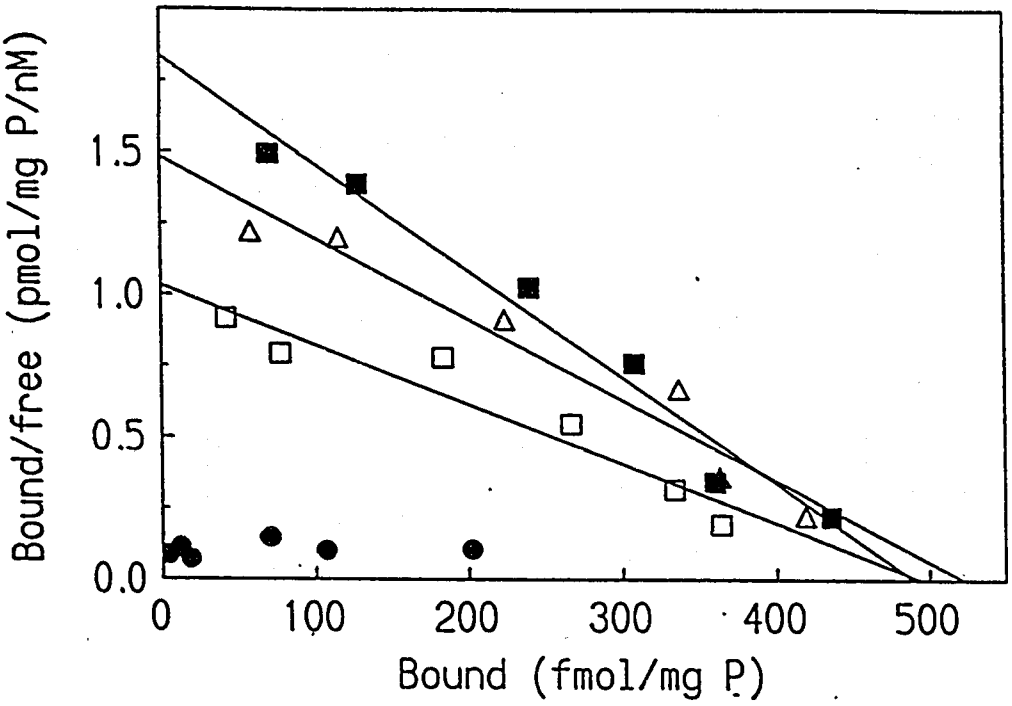


Fig.2. Scatchard analysis of specific binding of [^{3}H]paroxetine in membranes from rat diencephalon at increasing concentrations of sodium: 0 (●), 15 (□), 30 (△) and 60 (■) mM NaCl. Binding was determined over a concentration range of [^{3}H]paroxetine of 0.05 to 2.0 nM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 10 μM fluoxetine. Each point represents the mean ± SEM of four separate experiments performed in triplicate. The Bmax values were 513 ± 18 (15 mM NaCl), 537 ± 35 (30 mM NaCl) and 500 ± 36 fmol/mg protein (60 mM NaCl). The Kd values were 0.51 ± 0.02 (15 mM NaCl), 0.38 ± 0.05 (30 mM NaCl) and 0.28 ± 0.04 nM (60 mM NaCl).

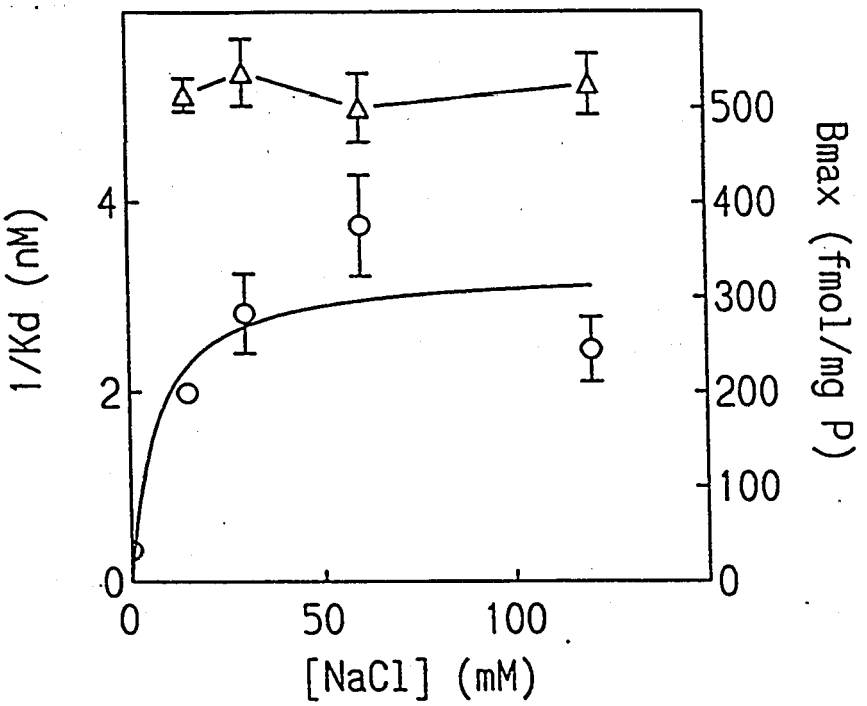


Fig. 3. Relationship of external sodium concentration and the affinity (1/Kd, ○) or density (Bmax, △) of specific binding of [^{3}H]paroxetine in membranes from rat diencephalon. Binding was determined over a concentration range of [^{3}H]paroxetine of 0.05 to 2.0 mM. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 10 μM fluoxetine. Data are transformed from saturation binding experiments presented in Fig. 2 and 1/Kd versus NaCl concentration data best fit a simple rectangular hyperbola ($r = 0.893$, $p < 0.025$). Each point represents the mean ± SEM of four separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol.

TABLE 1

Effect of sodium concentration on the kinetics of [^{3}H]5-HT uptake and [^{3}H]paroxetine binding in rat diencephalon. Each value represents the mean ± SEM of four ([^{3}H]paroxetine binding) or three ([^{3}H]5-HT uptake) separate experiments performed in triplicate.

	[^{3}H]Paroxetine binding		[^{3}H]5-HT uptake	
[NaCl] (mM)	Kd[a] (nM)	Bmax (fmol/mg P)	Km[b] (μM)	Vmax[c] (pmol/mg P/ 2 min)
15	0.51 ± 0.02	513 ± 18	0.43 ± 0.03	5.6 ± 1.0
30	0.38 ± 0.05	537 ± 35	0.50 ± 0.04	14.5 ± 2.6
60	0.28 ± 0.04	500 ± 36	0.28 ± 0.03	16.5 ± 2.6
120	0.43 ± 0.05	524 ± 32	0.21 ± 0.01	16.0 ± 1.3

[a] Significantly different (ANOVA, $f = 4.720$; $p < 0.022$)

[b] Significantly different (ANOVA, $f = 18.27$; $p < 0.002$)

[c] Significantly different (ANOVA, $f = 6.357$; $p < 0.017$)

required for the binding of one [^{3}H]paroxetine molecule.

The sodium requirement was also analysed by studying the binding of [^{3}H]paroxetine at a single concentration (0.25 or 0.4 nM, respectively) to membrane preparations and coronal sections from rat diencephalon at varying sodium concentrations. The results reported in Fig. 4 show that the binding increased significantly with increasing sodium concentrations to reach a plateau at 60 mM. The relationship between sodium concentration and [^{3}H]paroxetine binding in both cases was again best described by simple rectangular hyperbolas ($r = 0.956$; $p < 0.005$ for membrane preparation and $r = 0.769$; $p < 0.025$ for sections). The EC_{50} of sodium for the stimulation of [^{3}H]paroxetine binding in this experimental condition was approximately 5 mM and 10 mM in membranes and sections, respectively. The double reciprocal plot of the sodium dependence curve of [^{3}H]paroxetine in membrane preparation (Fig. 5) yielded a linear regression line ($r = 0.996$; $p < 0.005$) to be expected when one sodium is required for the binding of one [^{3}H]paroxetine molecule.

2. Sodium dependence of [^{3}H]5-HT uptake

Data presented in Figs. 6 and 7 demonstrate the effect of varying external sodium concentration on the kinetics of [^{3}H]5-HT uptake by synaptosomes of rat diencephalon. As sodium concentration in the medium increases, the uptake saturates at lower [^{3}H]5-HT concentrations and shows an increased initial transport rate (Vmax). Both the affinity (1/Km) and Vmax of [^{3}H]5-HT uptake showed significant changes due to varying sodium

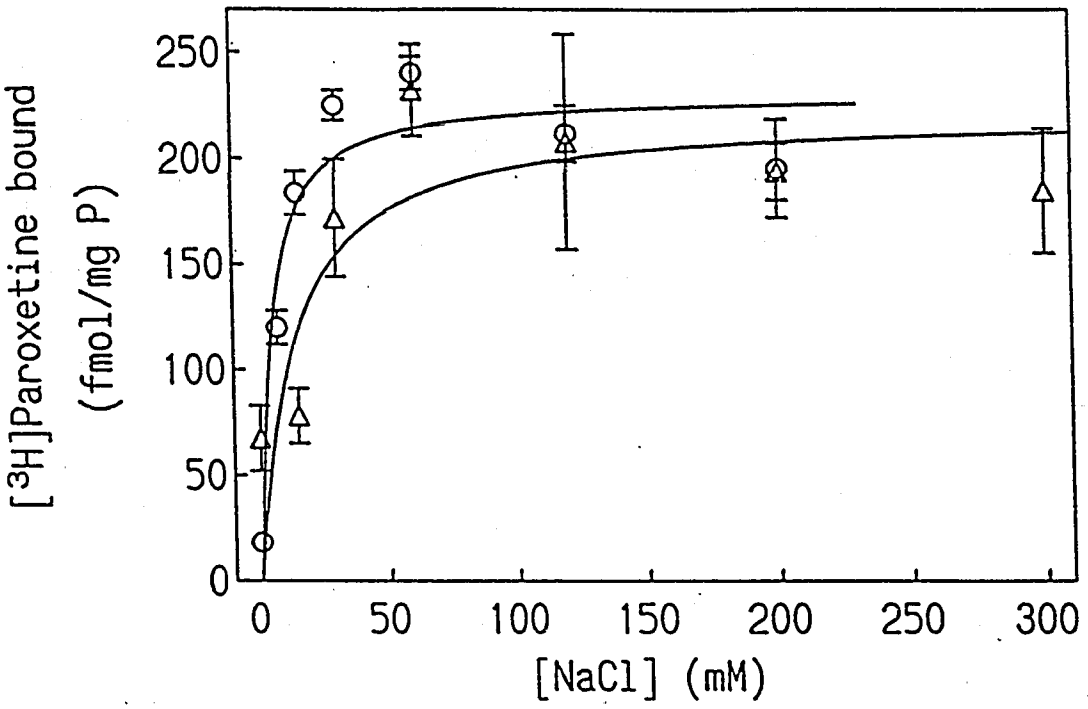


Fig. 4. Effect of increasing concentrations of sodium on specific binding of [^{3}H]paroxetine in membrane preparation (○) and coronal sections (△) from rat diencephalon. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 10 μM (homogenates) or 30 μM (sections) fluoxetine. Binding in homogenates was measured at 0.25 nM and in sections at 0.4 nM [^{3}H]paroxetine. Data best fit simple rectangular hyperbolas ($r = 0.956$; $p < 0.005$ for membranes and $r = 0.769$; $p < 0.025$ for sections). The EC_{50} of sodium was approximately 5 mM and 10 mM in membranes and sections respectively. Each point represents the mean ± SEM of four separate experiments performed in triplicate (homogenates) or duplicate (sections). Where error bars are not shown, the SEM was smaller than the size of the symbol.

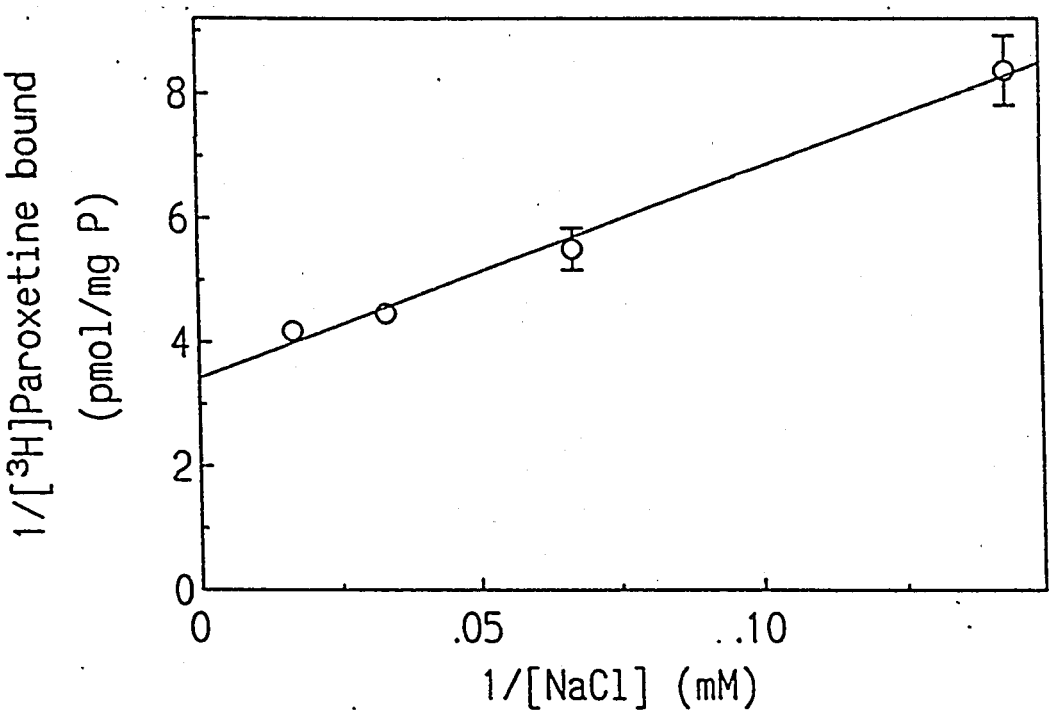


Fig. 5. Double reciprocal plot of specific binding of 0.25 nM [^{3}H]paroxetine in membranes from rat diencephalon and external sodium concentration ($r = 0.996$; $p < 0.005$ for the linear regression line). Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 10 μM fluoxetine. Each point represents the meab ± SEM of four separate experiments performed in triplicate. Data are transformed from the binding curve (points until saturation) shown in Fig. 4. Where error bars are not shown, the SEM was smaller than the size of the symbol.

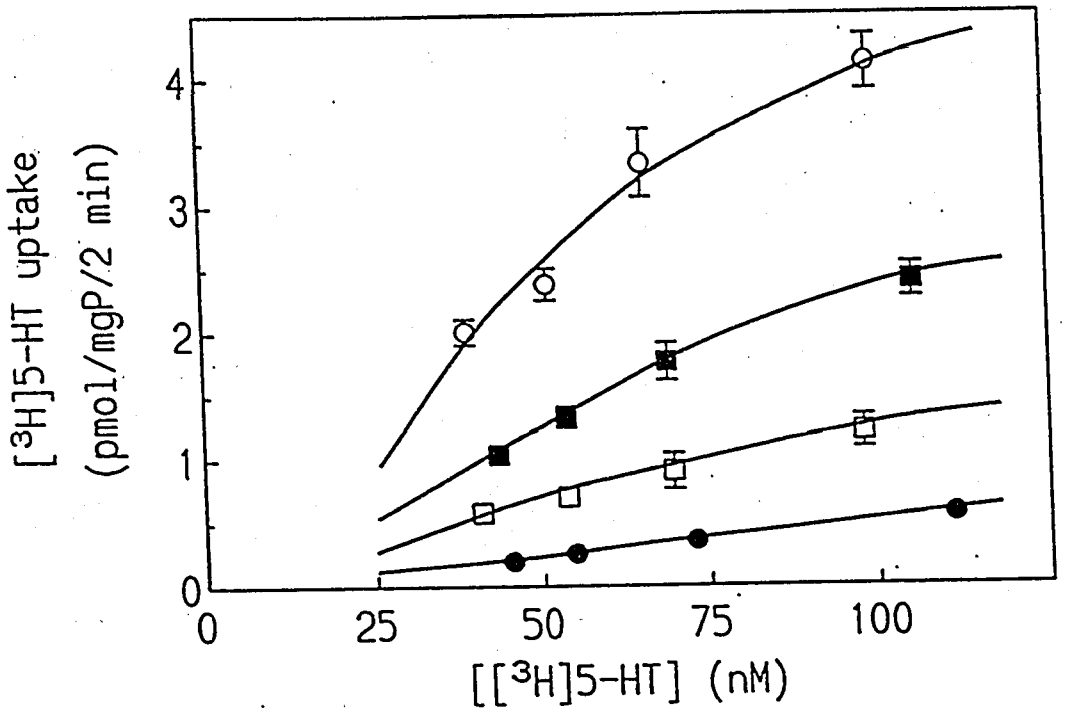


Fig. 6. Saturation curves for [^{3}H]5-HT uptake in synaptosomes from rat diencephalon at increasing concentrations of sodium: 0 (●), 15 (□), 30 (■) and 60 (○) mM NaCl. Uptake was determined over a concentration range of [^{3}H]5-HT of 40 to 100 nM. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Each point represents the mean ± SEM of three separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol.

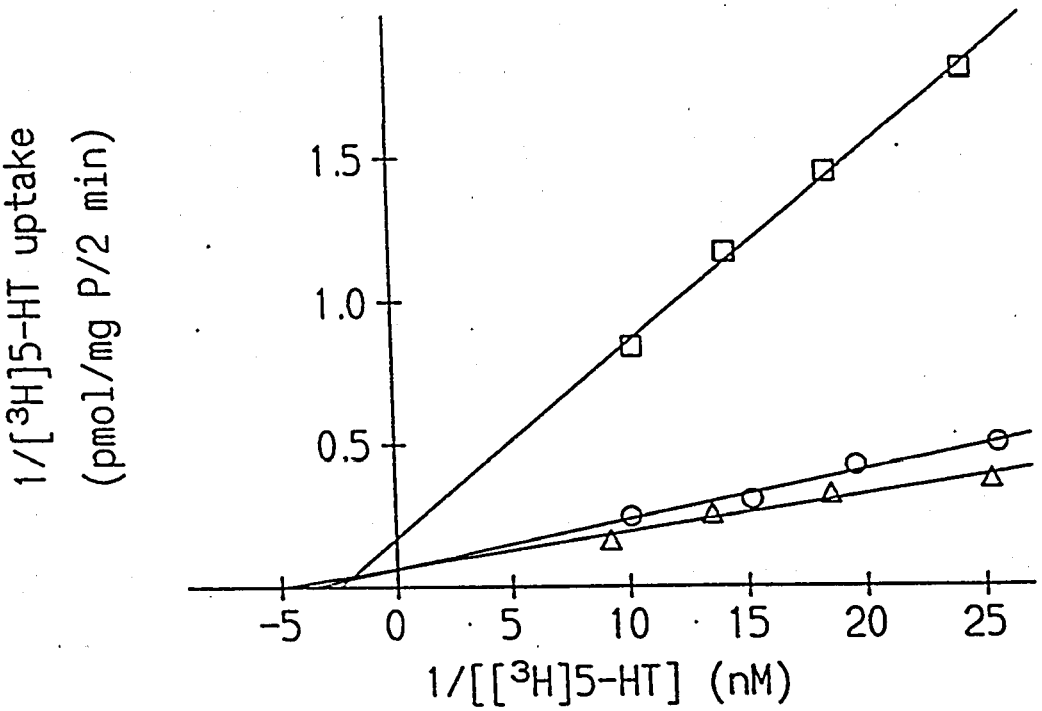


Fig. 7. Lineweaver-Burke plots of [^{3}H]5-HT uptake in synaptosomes from rat diencephalon at increasing concentrations of sodium: 15 (□), 60 (○) and 120 (△) mM NaCl. Uptake was determined over a concentration range of [^{3}H]5-HT of 40 to 100 nM. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Each point represents the mean ± SEM of three separate experiments performed in triplicate. The corresponding Vmax values were 5.6 ± 1.0, 16.5 ± 2.6 and 16.0 ± 1.3 pmol/mg protein/2 minutes. The corresponding Km values were 0.43 ± 0.03, 0.28 ± 0.03 and 0.21 ± 0.01 μM.

concentration (ANOVA, f = 54.87; $p < 0.001$ and f = 16.48; $p < 0.001$, respectively) (Figs. 7, 8, Table 1). The relationship between the external sodium concentration and the affinity of [^{3}H]5-HT for its transporter (1/Km) as well as the rate of uptake (Vmax) is reported in Fig 8. Both the affinity and transport rate increase by about 2 to 3 fold with elevating the sodium concentration from 15 mM to 120 mM. Points on both curves fit rectangular hyperbolas ($r = 0.967$; $p < 0.005$ for 1/Km and $r = 0.957$; $p < 0.01$ for Vmax) and suggest that one sodium ion is required for both binding and subsequent translocation of serotonin. A similar hyperbolic relationship was found between external sodium concentration and uptake of 50 nM [^{3}H]5-HT (Fig.9) ($r = 0.990$; $p < 0.005$) with an EC_{50} for sodium of approximately 35 mM. Double reciprocal plot of the sodium dependence curve of [^{3}H]5-HT uptake in this experimental condition yielded a linear regression line ($r = 0.998$; $p < 0.002$) (Fig. 10) suggesting again that one sodium ion is required for both binding and subsequent translocation of serotonin.

3. Effect of chronic treatment with antidepressants on [^{3}H]5-HT uptake

Chronic treatment with the tricyclic antidepressant desipramine (10 mg/kg/day for 21 days) caused no change in either the Vmax or the Kd of [^{3}H]5-HT uptake ($n = 6$) in comparison to saline-treated controls, in either cortical or hippocampal tissue (Fig. 11).

Chronic treatment with the selective 5-HT uptake inhibitor fluoxetine (5 mg/kg/day for 21 days) resulted in significant changes in the kinetic parameters of [^{3}H]5-HT uptake by crude cortical synaptosomal (P_2) fraction (Fig. 12) ($n = 6$). The Vmax in control rats was

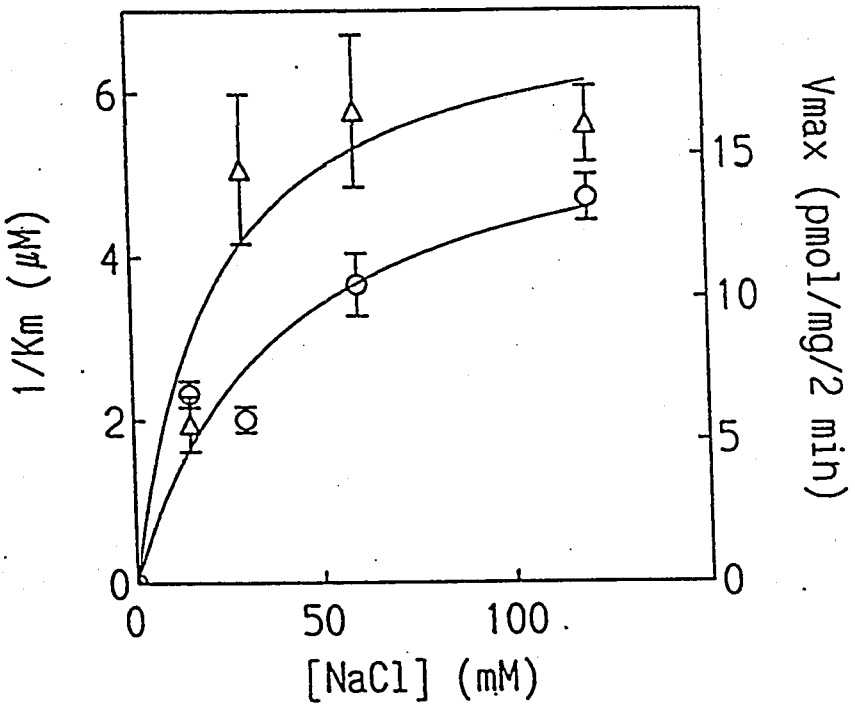


Fig. 8. Relationship of external sodium concentration and the affinity (1/Km, ○) or rate (Vmax, △) of [^{3}H]5-HT uptake by synaptosomes from rat diencephalon. Uptake was determined over a concentration range of [^{3}H]5-HT of 40 to 100 nM. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Data are transformed from kinetic experiments presented in Fig. 7 and best fit simple rectangular hyperbolas ($r = 0.967$; $p < 0.005$ for 1/Km and $r = 0.957$; $p < 0.01$ for Vmax). Each point represents the mean ± SEM of three separate experiments performed in triplicate.

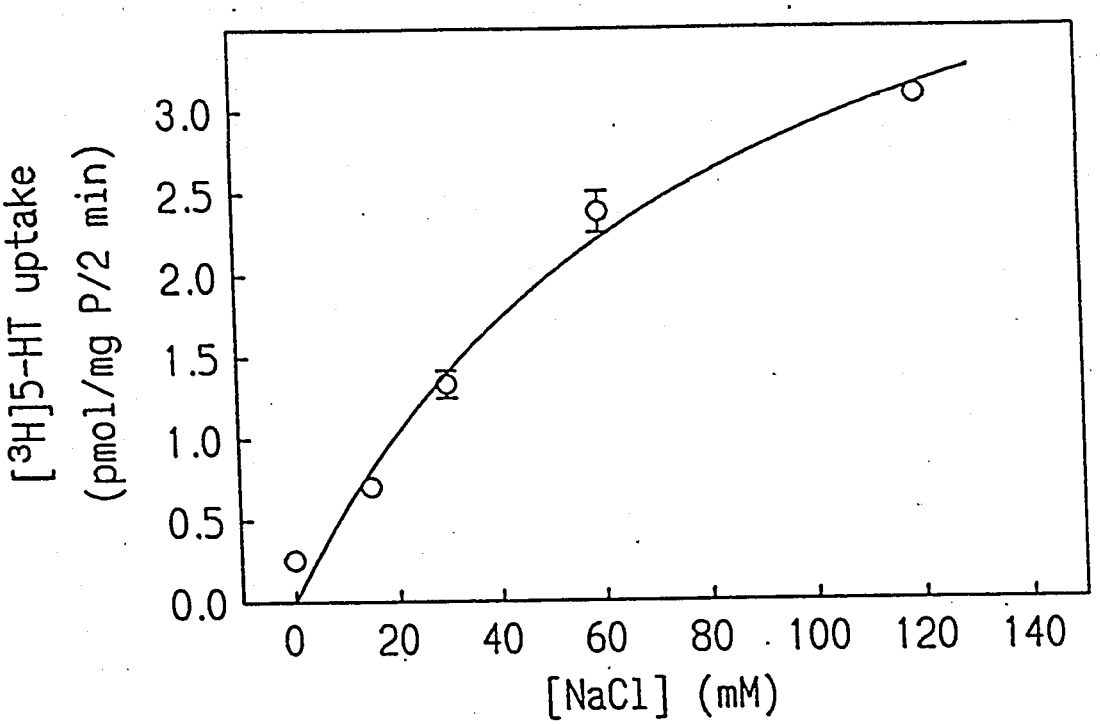


Fig. 9. Effect of increasing concentrations of sodium on net uptake of [^{3}H]5-HT in synaptosomes from rat diencephalon. Uptake was measured at 50 nM [^{3}H]5-HT. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Net uptake of [^{3}H]5-HT best fit a simple rectangular hyperbola ($r = 0.990$; $p < 0.005$). The EC_{50} for sodium was approximately 35 mM. Each point represents the mean ± SEM of three separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol.

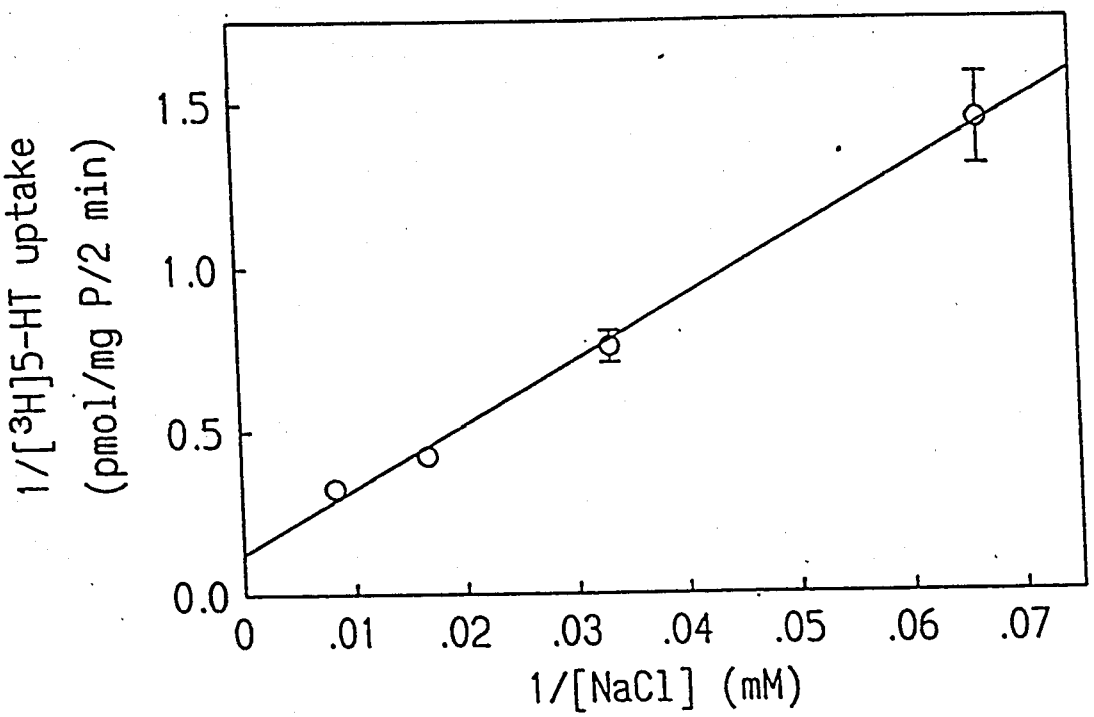


Fig. 10. Double reciprocal plot of net uptake of 50 nM [^{3}H]5-HT in synaptosomes from rat diencephalon and external sodium concentration ($r = 0.998$; $p < 0.002$) for the linear regression line). Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Data are transformed from the uptake curve shown in Fig. 9. Each point represents the mean ± SEM of three separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol.

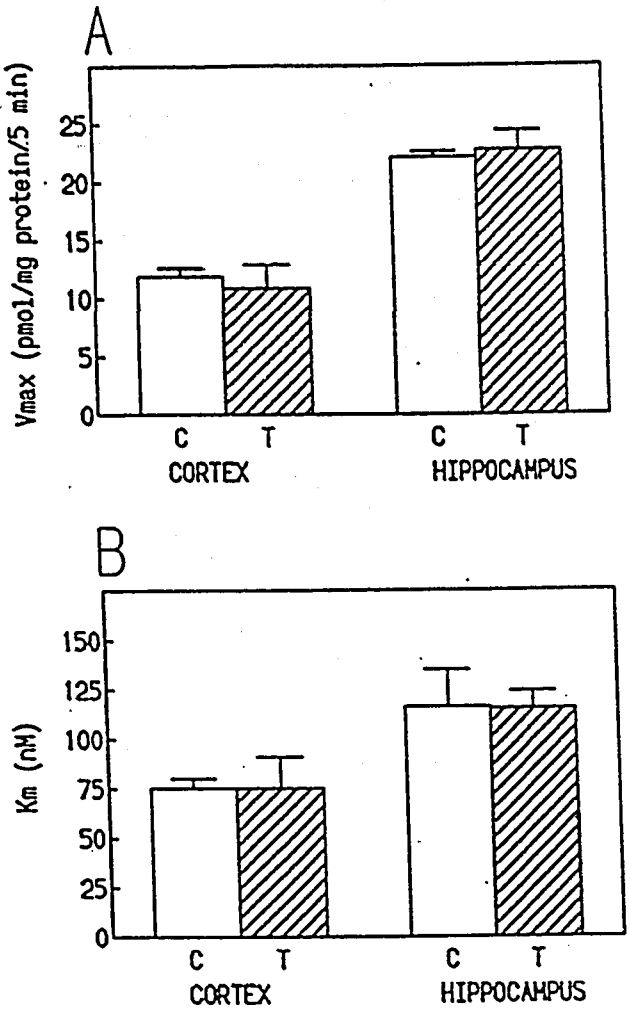


Fig. 11. Effect of chronic treatment with desipramine (10 mg/kg/day i.p. for 21 days) on kinetics (Vmax, *A*; Km, *B*) of [^{3}H]5-HT uptake in rat brain cortex and hippocampus. Uptake was determined over a concentration range of [^{3}H]5-HT of 40 to 100 nM. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Each bar represents the mean ± SEM of six separate experiments performed in triplicate. The Vmax values were 11.9 ± 0.7 (control cortex), 10.9 ± 2.0 (treated cortex), 22.1 ± 0.5 (control hippocampus) and 22.7 ± 1.7 pmol/mg protein/5 minutes (treated hippocampus). The Km values were 75 ± 5 (control cortex), 75 ± 16 (treated cortex), 116 ± 19 (control hippocampus) and 115 ± 9 nM (treated hippocampus).

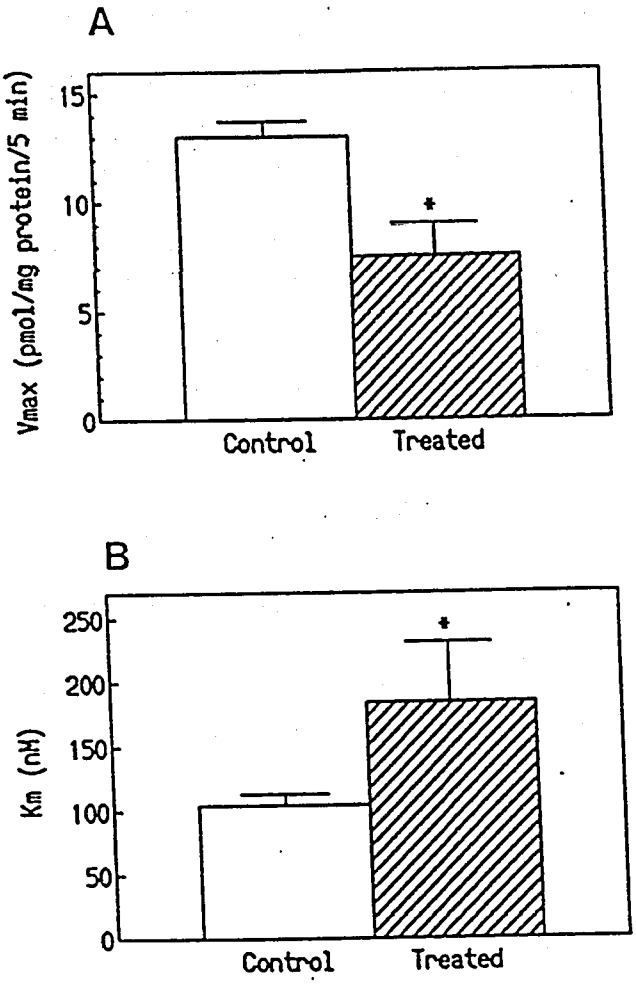


Fig. 12. Effect of chronic treatment with fluoxetine (5 mg/kg/day i.p. for 21 days) on kinetics (Vmax, *A*; Km, *B*) of [^{3}H]5-HT uptake in rat brain cortex. Uptake was determined over a concentration range of [^{3}H]5-HT of 40 to 100 nM. Net uptake was defined as the difference between uptake at 37°C (total) or 0°C (non-specific). Each bar represents the mean ± SEM of six separate experiments performed in triplicate. The Vmax values were 13.0 ± 0.7 (control) and 7.5 ± 1.5 pmol/mg protein/5 minutes (treated). The Km values were 104 ± 9 (control) and 184 ± 47 nM (treated). *Significantly different from the corresponding mean value in control animals ($p < 0.05$).

13.0 ± 0.7 pmol/mg protein and the Km 104 ± 9 nM. The Vmax was decreased in fluoxetine-treated animals to 7.5 ± 1.5 pmol/mg protein, a decrease of 42 % from the control value ($p < 0.05$). The affinity (1/Km) of [^{3}H]5-HT uptake was also decreased; Km of uptake was increased in treated animals to 184 ± 87 nM, an increase of 77 % from the control value ($p < 0.05$).

B. POSTRECEPTOR MECHANISMS: ANTIDEPRESSANTS AND PKC

1.1 PKC location: [^{3}H]PDBu binding in brain sections

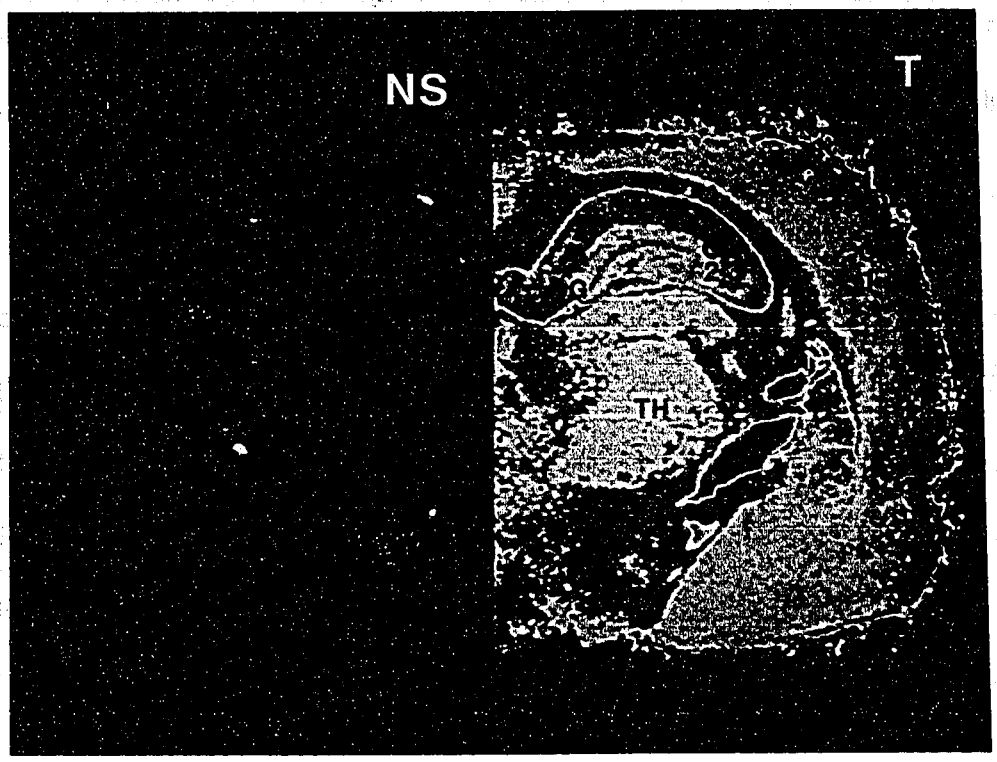
[^{3}H]PDBu binding (measured at 2.5 nM [^{3}H]PDBu) in sections from control rat diencephalon (5 to 6 mm anterior to the interaural line) revealed a high proportion of specific binding to total binding of [^{3}H]PDBu (94.3 ± 0.3 %) (n = 3). To assess potential effects of the presence of desipramine *in vitro*, sections of the same brain area from the same rats were incubated in the presence of 100 μM desipramine. The per cent specific binding remained high at 93.7 ± 0.7 % (n = 3).

1.2 Autoradiography of [^{3}H]PDBu binding to brain sections

Sections (5.2 mm anterior to the interaural line) from control brain (n = 3) incubated with [^{3}H]PDBu demonstrated a non-uniform distribution of label. Fig. 13 (*upper*) demonstrates the distribution of the [^{3}H]PDBu label in a section from a control brain. Highest levels of label were found in the outer 3 layers of the parietal cortex, and in the CA_1, CA_2, CA_3 and

Fig. 13. Autoradiographic visualization of [^{3}H]PDBu binding in coronal sections of rat diencephalon (5.2 mm anterior to interaural line). *A.* Total (*right, T*) and non-specific binding (*left, NS*) of [^{3}H]PDBu in adjacent brain sections from a control rat. *B.* Total (*right, T*) and non-specific binding (*left, NS*) of [^{3}H]PDBu in adjacent brain sections from a rat treated 2 hours before death with desipramine (10 mg/kg i.p.). Increased redness corresponds to higher levels of grains associated with binding of [^{3}H]compounds. Binding was measured at 2.5 nM [^{3}H]PDBu. Brain structures were identified by using the atlas of Paxinos and Watson (1986). CA1, CA1 field of hippocampus; CA2,3, CA2and CA3 fields of hippocampus; CX1-3, cortical layers 1 to 3; DG, dentate gyrus; H, hypothalamus; TH, thalamus.

A



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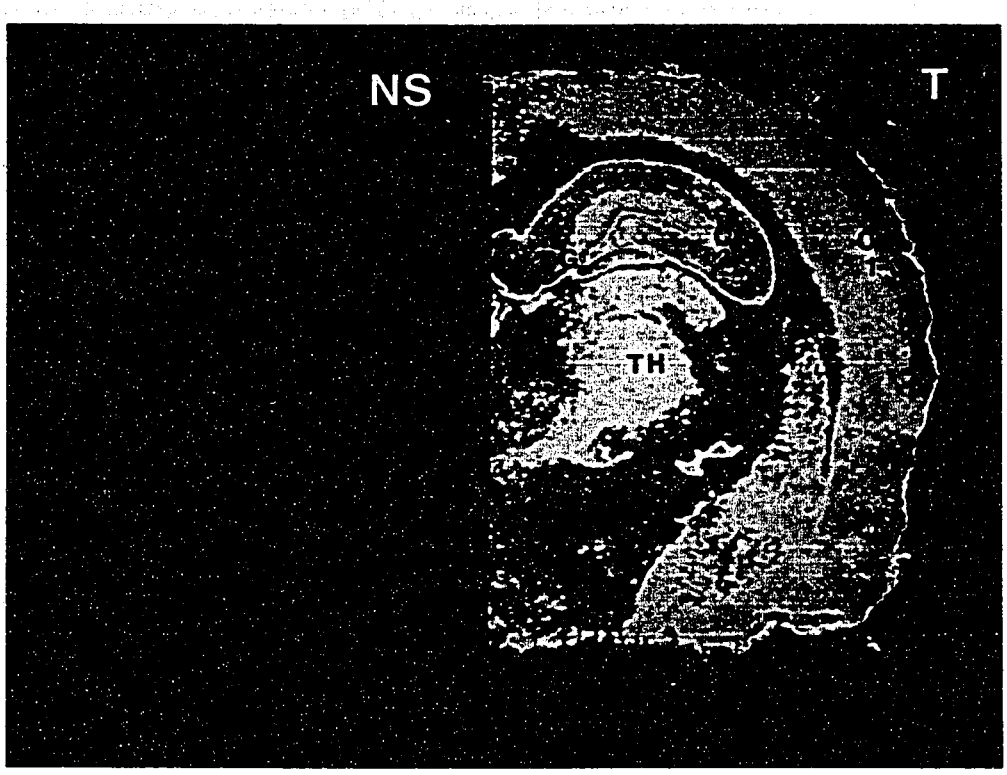

dentate gyrus regions of the hippocampus (Table 2). Intermediate levels of the label were found in the inner 3 layers of the parietal cortex and the striatum. Lowest levels of binding were found in the hypothalamus, the thalamus and the corpus callosum.

Another group of animals (n = 3) was treated 2 hours before death with 10 mg/kg desipramine in order to assess the effect of acute treatment with this drug on [^{3}H]PDBu binding in the brain. An autoradiogram generated from a treated rat (Fig. 13, *lower*) shows the same distribution of label as in the control brain (Fig. 13, *upper*) although the label is weaker in all areas. [^{3}H]PDBu binding in DMI-treated rats was significantly lower than that in controls in both the parietal cortex (layers 4 to 6) ($p < 0.005$) and in the striatum ($p < 0.05$) (Table 2).

2. PKC location: [^{3}H]PDBu binding in subcellular fractions of brain

2.1 Time course of [^{3}H]PDBu binding

Fig. 14 shows the time course of specific binding of [^{3}H]PDBu in the soluble fraction of cortical tissue. The incubation times tested ranged from 15 minutes to 2.5 hours. As incubation time was increased, [^{3}H]PDBu binding increased as a simple rectangular hyperbola ($r = 0.996$; $p < 0.005$) (n = 1) with highest binding of [^{3}H]PDBu occurring at 2 hours (66.2 pmol/mg protein). All subsequent [^{3}H]PDBu binding assays were thus performed using a 2 hour incubation period.

Table 2

Effect of acute treatment with DMI (10 mg/kg i.p. one hour before death) on the specific binding of 2.5 nM [^{3}H]PDBu in rat brain regions. Each value represents the mean ± SEM of three separate experiments performed in duplicate.

Brain area	Control (pmol/mg protein)	DMI (pmol/mg protein)
Frontal cortex	18.1 ± 0.5	16.6 ± 1.2
Parietal cortex (layers 1 - 3)	28.3 ± 0.7	25.7 ± 1.8
Parietal cortex (layers 4 - 6)	22.4 ± 0.1	19.4 ± 0.5[a]
Striatum	23.3 ± 0.3	21.3 ± 0.6[b]
Dentate gyrus	21.1 ± 0.3	18.7 ± 1.3
CA1 field of hippocampus	24.1 ± 1.1	22.8 ± 1.8
CA2 and 3 fields of hippocampus	27.9 ± 1.2	23.5 ± 1.2
Hypothalamus	8.6 ± 0.8	8.3 ± 0.8
Piriform cortex	29.8 ± 1.0	27.1 ± 0.9

[a]Significantly different from corresponding mean control value ($p < 0.005$)

[b]Significantly different from corresponding mean control value ($p < 0.05$)

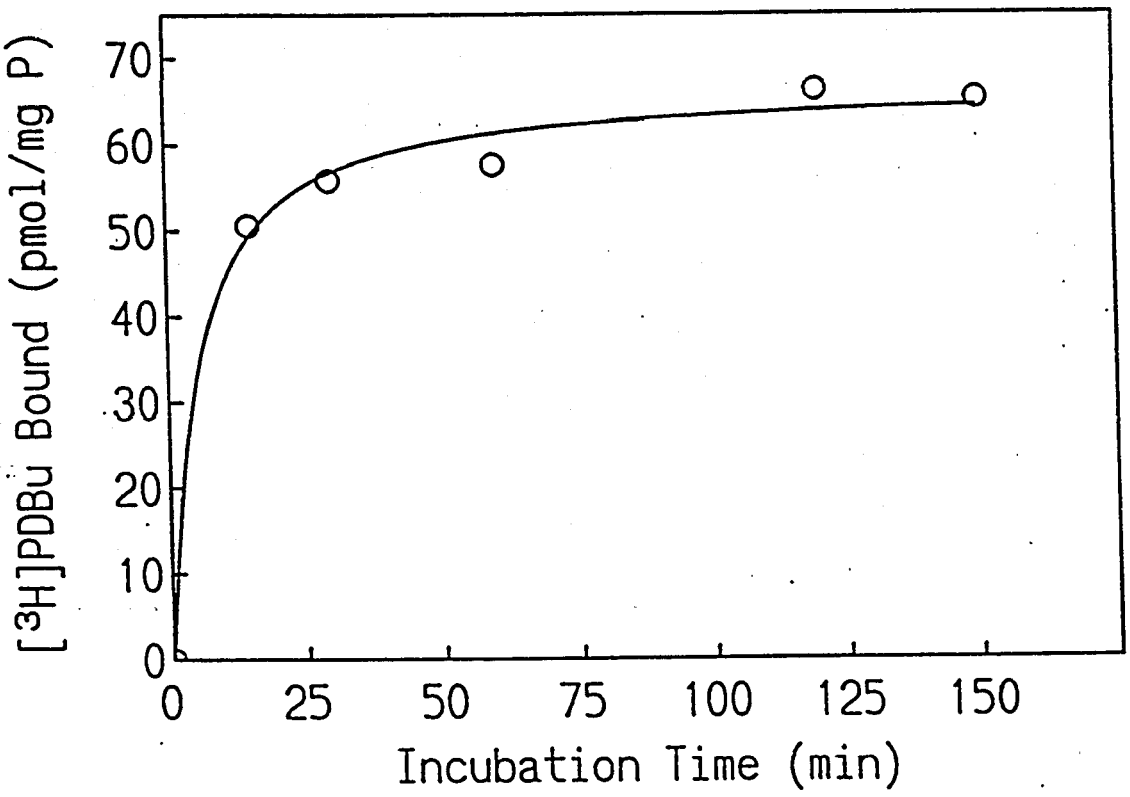

Fig. 14. Time course of specific binding of [^{3}H]PDBu in soluble fraction of rat brain cortex. Binding was determined at 2.5 nM [^{3}H]PDBu. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 2 μM PDBu. Data best fits a simple rectangular hyperbola ($r = 0.996$; $p < 0.005$). Each point represents the result of one experiment performed in duplicate.

2.2 [^{3}H]PDBu binding at different protein concentrations

Specific binding of [^{3}H]PDBu for both soluble and particulate fractions of cortical tissue at different protein concentrations is depicted in Fig. 15. As protein concentration in assay tubes of both fractions was increased, [^{3}H]PDBu binding increased as simple rectangular hyperbolas ($r = 0.997$; $p < 0.005$ for soluble and $r = 0.967$; $p < 0.005$ for particulate fractions) ($n = 1$). In subsequent experiments, the concentrations of proteins used in the assay were about 30 μg per tube for the soluble fraction and about 22 μg per tube for the particulate fraction, as these concentrations corresponded to [^{3}H]PDBu binding values which were still on the linear portion of the curve.

2.3 Competition curve of [^{3}H]PDBu binding

A competition curve (log scale) of the specific binding of [^{3}H]PDBu in the soluble fraction of cortical tissue is depicted in Fig. 16 ($r = 0.998$; $p < 0.005$) ($n = 4$). As the concentration of cold PDBu was increased from 0 to 20 μM, [^{3}H]PDBu binding decreased to less than 1 % of the total binding occurring in the absence of cold PDBu. The IC_{50} value of the curve (concentration of cold PDBu at which 50 % of [^{3}H]PDBu binding was abolished) was 8.7 nM PDBu. A concentration of 2 μM PDBu, corresponding to about 100 times the Kd of [^{3}H]PDBu binding (2.5 nM), was chosen to be used in subsequent experiments to define non-specific binding, as this concentration abolished over 98 % of total [^{3}H]PDBu binding.

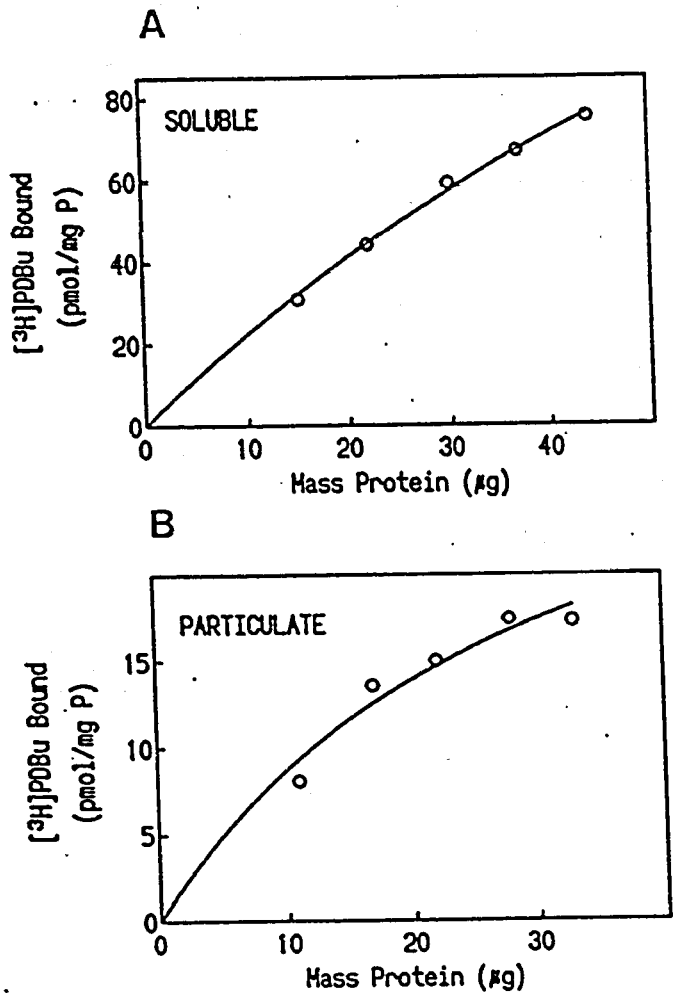

Fig. 15. Specific binding of [^{3}H]PDBu in soluble (*A*) and particulate (*B*) fractions of rat brain cortex at different protein concentrations. Binding was determined at 2.5 nM [^{3}H]PDBu. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 2 μM cold PDBu. Data best fit simple hyperbolic hyperbolas ($r = 0.997$; $p < 0.005$ for soluble fraction and $r = 0.967$; $p < 0.005$ for particulate fraction). Each point represents the result of one experiment performed in duplicate.

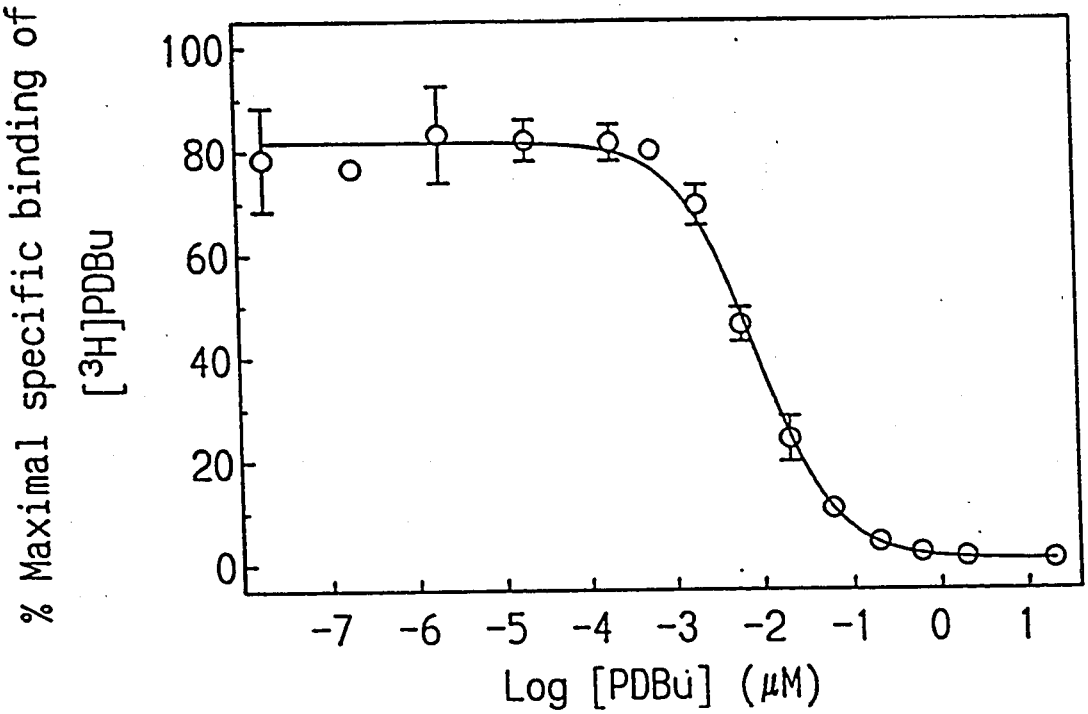

Fig. 16. Competition curve of [^{3}H]PDBu binding in soluble fraction of rat brain cortex: displacement of [^{3}H]PDBu binding by increasing concentrations of cold PDBu. Binding was determined at 2.5 nM [^{3}H]PDBu. Cold PDBu was present in concentrations ranging from 0 to 20 μM. Data best fit a single site competition curve (log scale) ($r = 0.998$; $p < 0.005$) with an IC_{50} value of 8.7 nM PDBu. Each point represents the mean ± SEM of four separate experiments performed in duplicate.

2.4 Subcellular distribution of [^{3}H]PDBu binding

Fig. 17 demonstrates the distribution of PKC location between the soluble and particulate fractions of cortical and hippocampal tissue. In the cortex [^{3}H]PDBu binding amounted to 65.8 ± 3.73 pmol/mg protein in the soluble fraction and 12.1 ± 1.01 pmol/mg protein in the particulate fraction (n = 4). In the hippocampus [^{3}H]PDBu binding was not significantly different from that in the cortex and amounted to 55.3 ± 9.51 in the soluble and 9.28 ± 1.21 pmol/mg protein in the particulate fraction (n = 4). The percent distribution of PKC between the 2 fractions in both tissues was about 85 % in the soluble fraction and 15 % in the particulate fraction. The specific binding of [^{3}H]PDBu amounted to between 95 and 99 % of total binding and 80 to 90 % of total binding in the soluble and particulate fractions, respectively.

2.5 Effect of acute DOI treatment on [^{3}H]PDBu binding

Acute treatment of rats (n = 4) with the 5-HT_2 receptor agonist DOI (10 mg/kg i.p. one hour before death) produced a significant decrease in [^{3}H]PDBu binding in both the soluble and the particulate fractions of the cortex ($p < 0.005$, $p < 0.05$, respectively) as compared to controls (Fig. 18). Binding amounted to 41.3 ± 2.89 as compared to 65.5 ± 3.73 pmol/mg protein in controls in the soluble fraction, and to 8.76 ± 0.65 as compared to 12.1 ± 1.01 pmol/mg protein in controls in the particulate fraction. There was no significant change in [^{3}H]PDBu binding in either fraction of the hippocampus following DOI pretreatment.

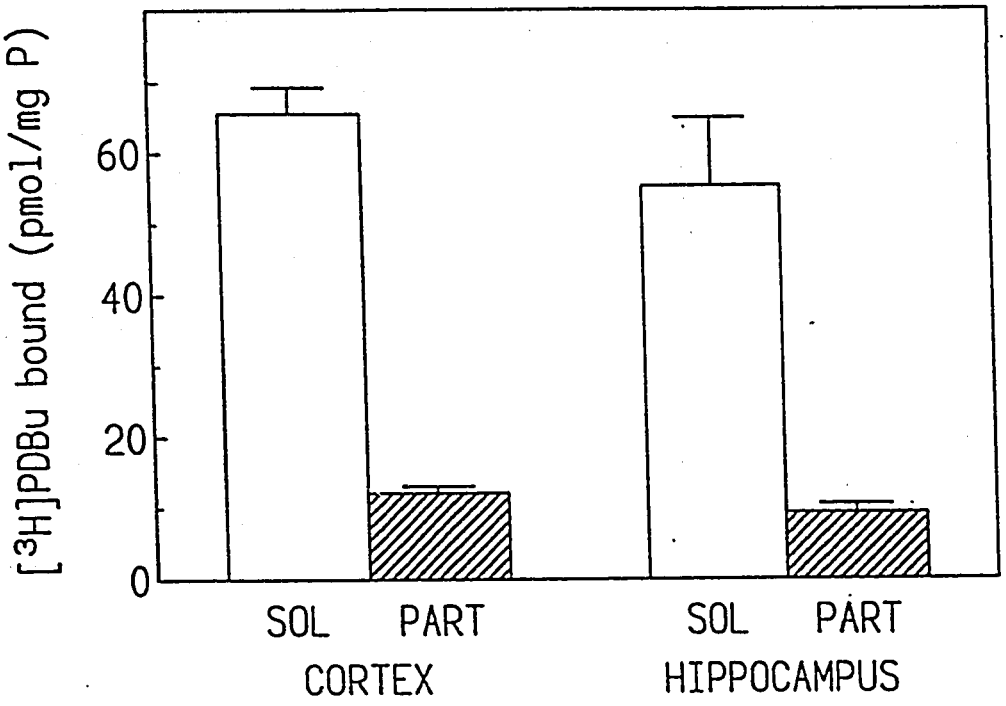

Fig. 17. Specific binding of [^{3}H]PDBu in soluble and particulate fractions of rat brain cortex and hippocampus. Binding was determined at 2.5 nM [^{3}H]PDBu. Specific binding was defined as the difference between binding in the absence (total) and presence (non-specific) of 2 μM cold PDBu. Each bar represents the mean ± SEM of four separate experiments performed in triplicate.

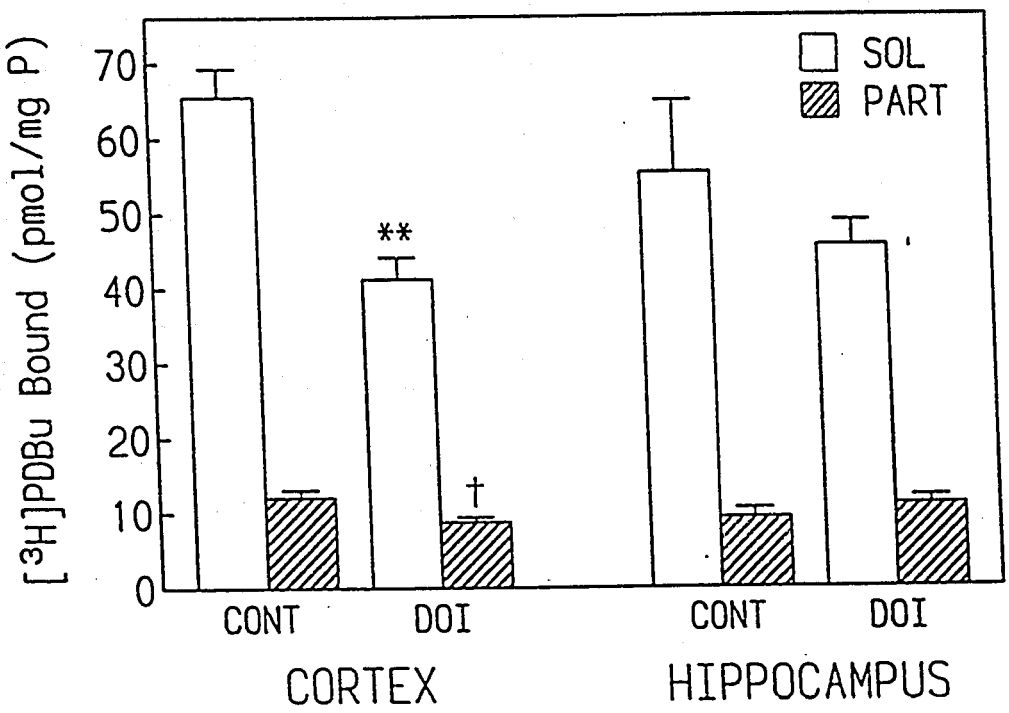

Fig. 18. Effect of acute treatment with DOI (10 mg/kg i.p. one hour before death) on specific binding of [^{3}H]PDBu in soluble and particulate fractions of rat brain cortex and hippocampus compared with controls. Binding was determined at 2.5 nM [^{3}H]PDBu. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 2 μM cold PDBu. Each bar represents the mean ± SEM of four separate experiments performed in triplicate (**$p < 0.005$; † $p < 0.05$).

2.6 Effect of chronic desipramine treatment on [^{3}H]PDBu binding

Chronic treatment of rats (n = 6) with the tricyclic antidepressant desipramine (10 mg/kg/day i.p. for 21 days) produced no change in [^{3}H]PDBu binding in either the soluble or the particulate fractions of cortical or hippocampal tissue of treated animals compared to saline-treated controls (Fig.19).

3. PKC activity in subcellular fractions of brain

3.1 PKC activity at different protein concentrations

PKC activity in both soluble and particulate fractions of cortical tissues at different protein concentrations is depicted in Fig. 20. As protein content in assay tubes of both fractions was increased, PKC activity increased to peaks occurring at about 8 and 7 μg in the soluble and particulate fractions respectively, and then abruptly decreased with higher concentrations of protein (n = 3). In subsequent experiments, the concentrations of proteins used in the assay were between 5 and 6 μg per tube, as these concentrations corresponded to PKC activities which were still on the linear (ascending) portions of the curves.

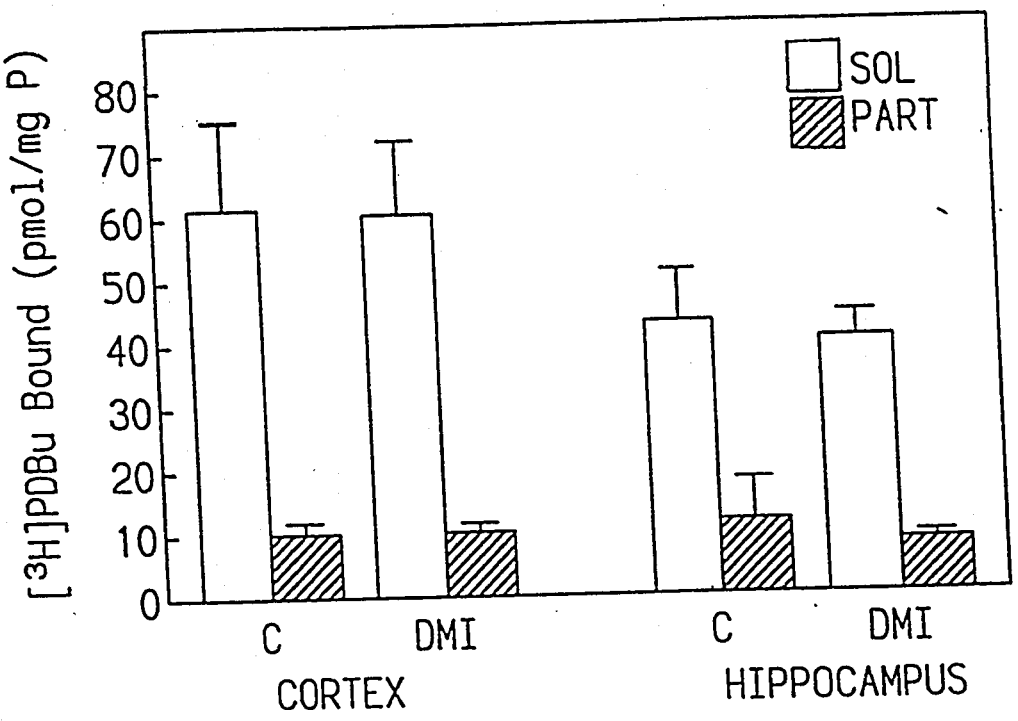

Fig. 19. Effect of chronic treatment with desipramine (10 mg/kg/day i.p. for 21 days) on specific binding of [^{3}H]PDBu in soluble and particulate fractions of rat brain cortex and hippocampus compared with controls (equal volume of 0.9 % saline each day for 21 days). Binding was determined at 2.5 nM [^{3}H]PDBu. Specific binding was defined as the difference between binding in the absence (total) or presence (non-specific) of 2 μM cold PDBu. Each bar represents the mean ± SEM of six separate experiments performed in triplicate.

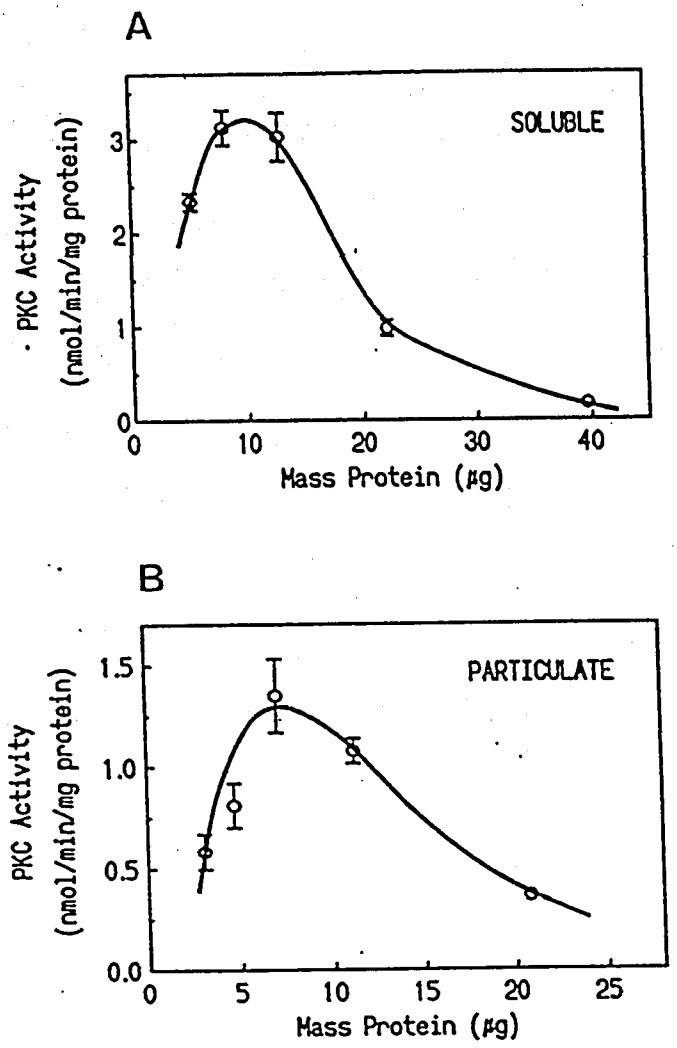

Fig. 20. PKC activity in soluble (*A*) and particulate (*B*) fractions of rat brain cortex at different protein concentrations. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each point represents the mean ± SEM of three separate experiments performed in duplicate.

3.2 Calcium and phospholipid dependence of PKC activity and specificity of target phosphorylation

When calcium acetate and phosphatidylserine (2 necessary cofactors for phosphorylation catalysed by PKC) were omitted from the incubation medium of the PKC activity assay and replaced by 3 mM EGTA, the enzyme activity was substantially decreased, to 5.0 % and 4.6 % of the control values in the soluble and to 23.1 % and 14.1 % of the control values in the particulate fractions of the cortex and hippocampus, respectively (Fig. 22) (n = 1). When the PKC-specific target peptide was omitted in the incubation medium, the enzyme activity was also substantially decreased, to 2.7 % and 3.9 % of the control values in the soluble and 4.4 % and 9.0 % of the control values in the particulate fractions of the cortex and hippocampus, respectively (Fig. 21).

3.3 Distribution of PKC activity in subcellular fractions

Fig. 22 demonstrates the distribution of PKC activity between the soluble and particulate fractions of control cortical and hippocampal tissue (n = 6). Activity did not differ significantly from one tissue to the other. In the cortex, PKC activity amounted to 3.49 ± 0.12 and 1.36 ± 0.08 nmol/min/mg protein in the soluble and particulate fractions respectively, and in the hippocampus activity amounted to 4.35 ± 0.53 and 1.48 ± 0.15 nmol/min/mg protein in the soluble and particulate fractions respectively. In the cortex, the per cent of total cellular PKC activity amounted to 71.9 ± 1.45 % in the soluble and 28.1 ± 1.45 % in the particulate fractions, while in the hippocampus 74.3 ± 1.16 % of

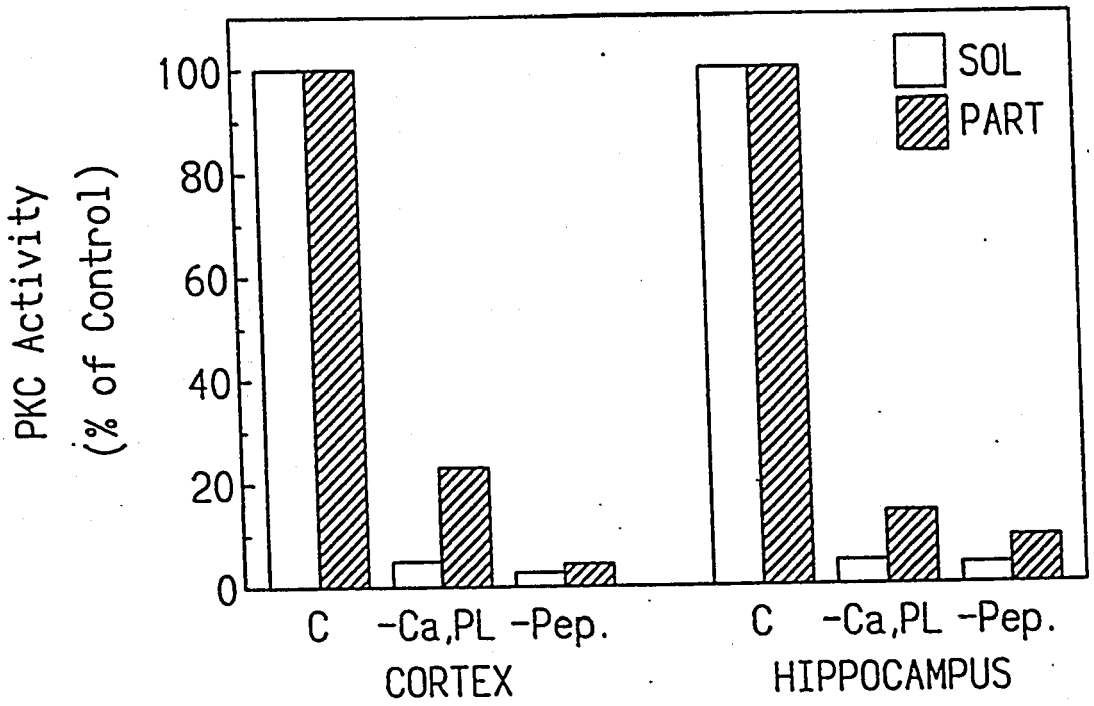

Fig. 21. Effect of absence of calcium (calcium acetate) and phospholipid (Lα phosphatidyl-L-serine) or of PKC-specific target peptide on the activity of PKC in soluble and particulate fractions of rat brain cortex and hippocampus. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the results of one experiment performed in duplicate.

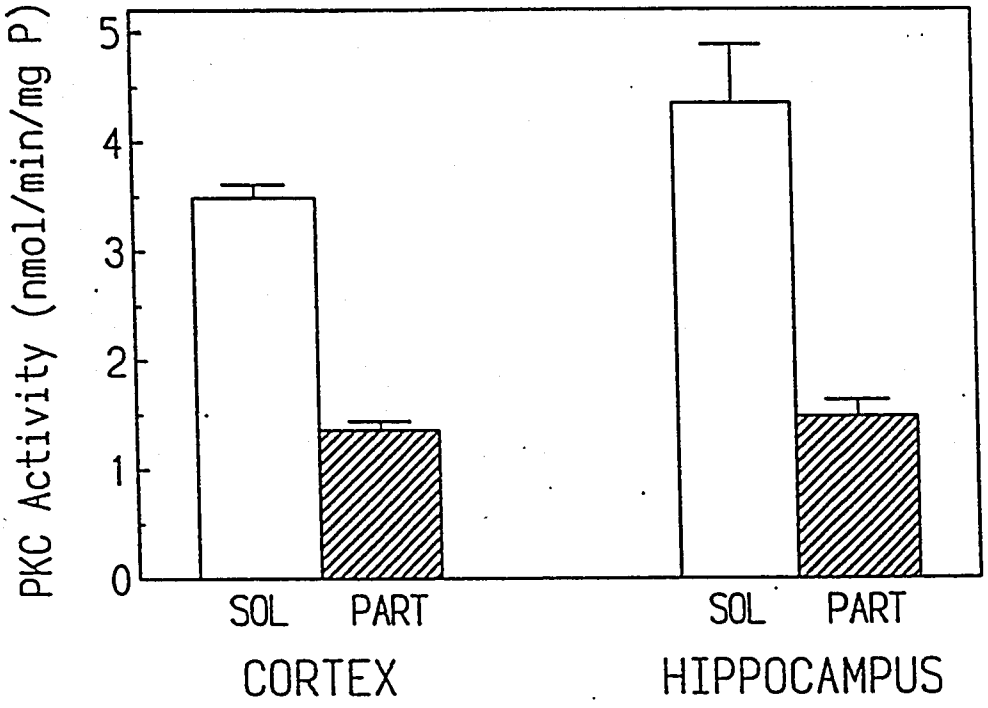

Fig. 22. PKC activity in soluble and particulate fractions of rat brain cortex and hippocampus. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of six separate experiments performed in duplicate.

activity was in the soluble fraction and 25.7 ± 1.16 % was in the particulate fraction.

3.4 Effect of acute DOI treatment on PKC activity

Treatment of rats with one dose of DOI (10 mg/kg i.p. one hour before death) resulted in significant decreases in PKC activity in both fractions of the cortex ($p < 0.005$ in both fractions) ($n = 4$) (Fig. 23). Activity decreased from 3.49 ± 0.12 to 2.68 ± 0.12 in the soluble and from 1.36 ± 0.08 to 0.86 ± 0.10 nmol/min/mg protein in the particulate fractions. Distribution of PKC activity in treated animals was 75.6 ± 2.95 % in the soluble and 24.4 ± 2.95 % in the particulate fractions. In the hippocampus, there was a decrease in particulate fraction activity from 1.48 ± 0.15 to 0.83 ± 0.03 nmol/min/mg protein ($p < 0.05$) , with a resulting distribution of activity of 83.1 ± 1.06 % and 16.9 ± 1.06 % in the soluble and particulate fractions respectively.

3.5 Effect of acute antidepressant treatment on PKC activity

Fig. 24 shows the effect of one dose of desipramine (10 mg/kg i.p. one hour before death) on PKC activity ($n = 4$). Particulate fraction activity in both tissues of treated animals decreased significantly ($p < 0.05$): from 1.36 ± 0.08 to 0.91 ± 0.17 nmol/min/mg protein in the cortex and from 1.48 ± 0.15 to 0.85 ± 0.01 nmol/min/mg protein in the hippocampus. In treated animals the distribution of PKC activity was 76.3 ± 3.29 % and 23.7 ± 3.29 % in the soluble and particulate fractions of the cortex respectively, and 79.4 ± 1.26 % and 20.6 ± 1.26 % in the soluble and particulate

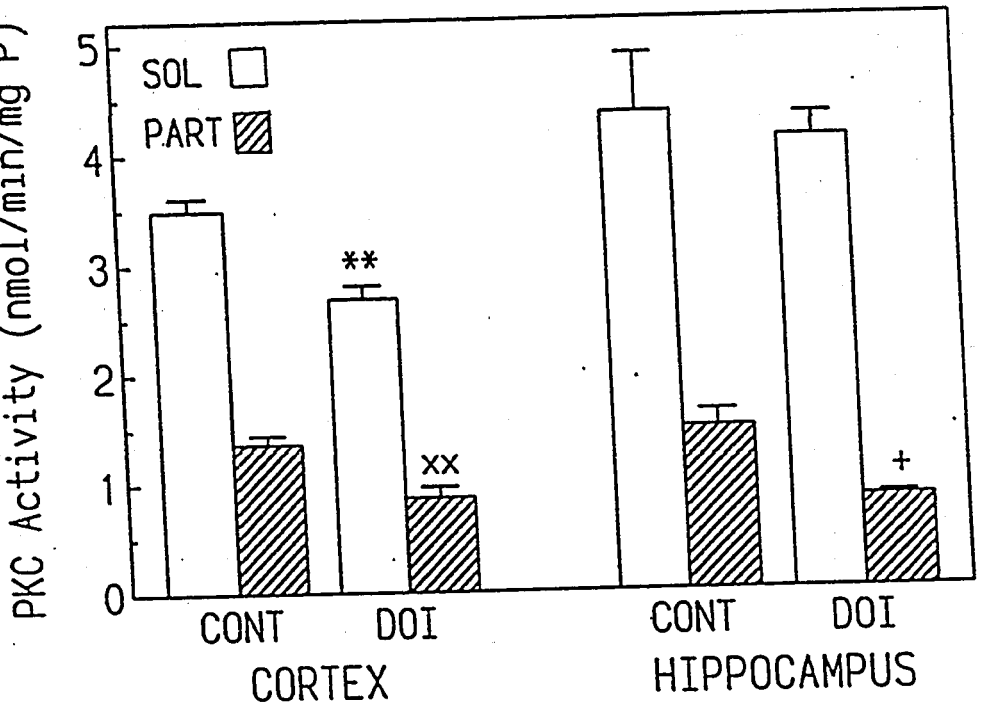

Fig. 23. Effect of acute treatment with DOI (10 mg/kg i.p. one hour before death) on PKC activity in soluble and particulate fractions of rat brain cortex and hippocampus. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of four separate experiments performed in duplicate (**$p < 0.005$; xx $p < 0.005$; + $p < 0.05$).

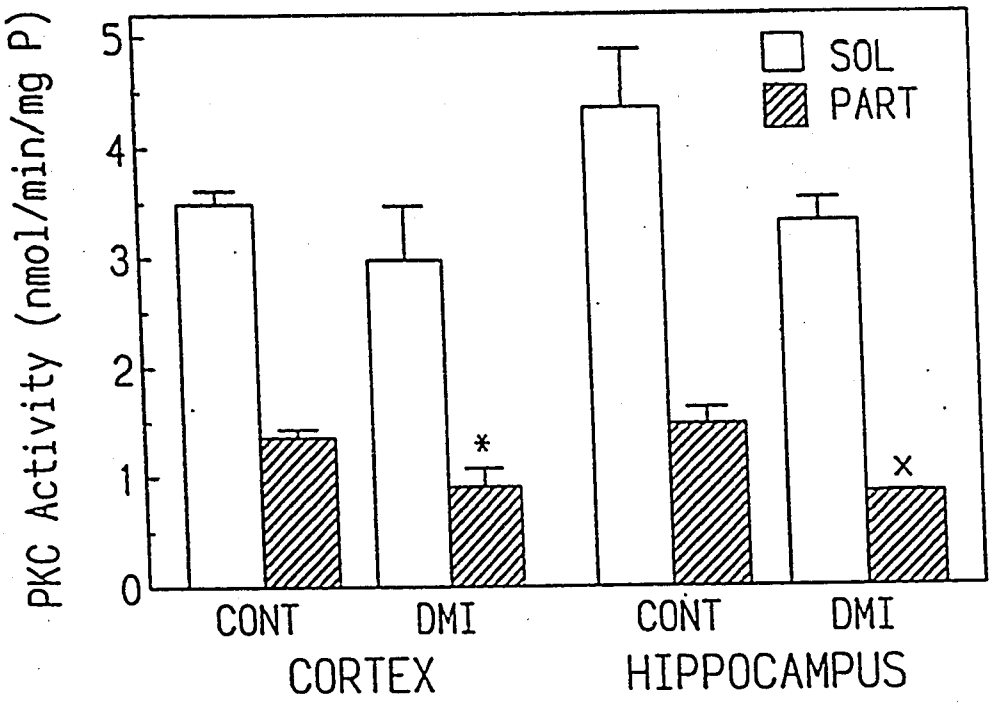

Fig. 24. Effect of acute treatment with desipramine (10 mg/kg i.p. one hour before death) on PKC activity in soluble and particulate fractions of rat brain cortex and hippocampus. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of four separate experiments performed in duplicate (*$p < 0.05$; x $p < 0.05$).

fractions of the hippocampus respectively.

The effect of one dose of fluoxetine (5 mg/kg i.p. one hour before death) on PKC activity is depicted in Fig. 25. ($n = 4$). Cortical PKC activity was decreased in both cellular fractions in treated animals ($p < 0.05$ in soluble; $p < 0.005$ in particulate), from 3.49 ± 0.12 to 2.68 ± 0.11 nmol/min/mg protein in the soluble and from 1.36 ± 0.08 to 0.71 ± 0.12 nmol/min/mg protein in the particulate fractions. Distribution of PKC activity in treated animals was 79.4 ± 2.7 % and 20.6 ± 2.7 % in the soluble and particulate fractions respectively. In the hippocampus, there was a significant decrease of activity in the particulate fraction only ($p < 0.05$), from 1.48 ± 0.15 to 0.95 ± 0.05 nmol/min/mg protein , resulting in a tissue distribution of activity of 81.0 ± 1.13 % in the soluble and 19.0 ± 1.13 % in the particulate fractions.

3.6. Effect of chronic desipramine treatment on PKC activity

Chronic treatment of rats ($n = 5$) with desipramine (10 mg/kg/day i.p. for 21 days) produced a significant decrease in basal PKC activity in the soluble fraction of the cortex, to 1.35 ± 0.12 nmol/min/mg protein compared to saline-treated controls (2.17 ± 0.11 nmol/min/mg protein) ($p < 0.005$) (Fig. 26). Distribution of PKC activity in soluble and particulate fractions, respectively, was 72.6 ± 1.72 % and 27.4 ± 1.72 % in saline-treated controls and 71.1 ± 2.25 % and 28.9 ± 2.25 % in desipramine-treated animals. Treatment with desipramine did not alter the activity in either cellular fraction of the cortex following acute challenge with DOI (10 mg/kg i.p. one hour before death; $n = 5$).

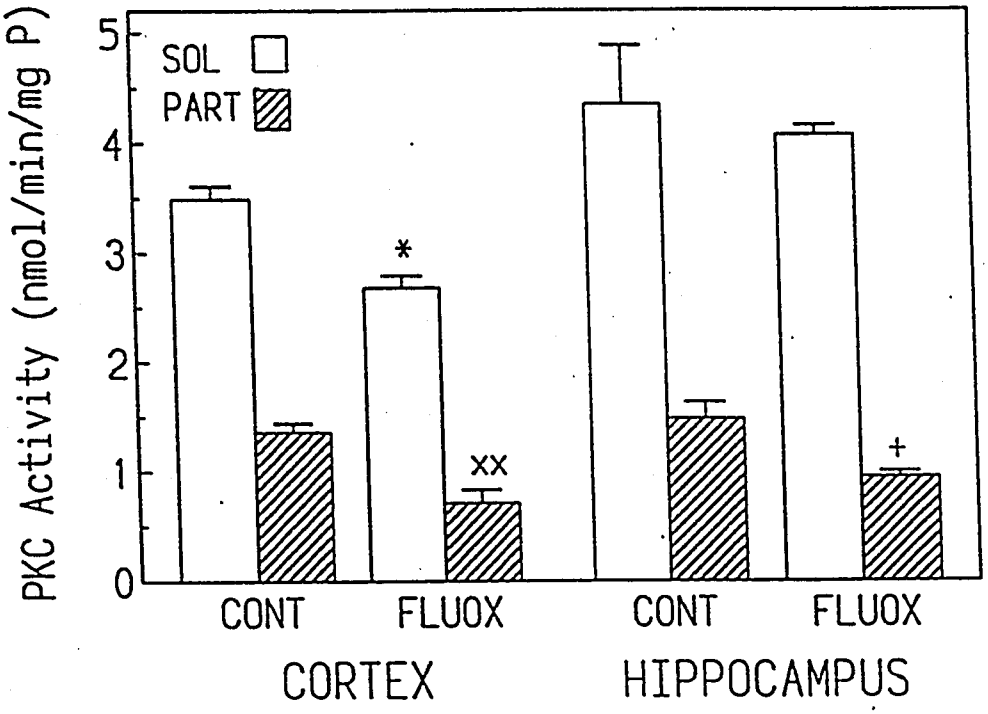

Fig. 25. Effect of acute treatment with fluoxetine (5 mg/kg i.p. one hour before death) on PKC activity in soluble and particulate fractions of rat brain cortex and hippocampus. Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of four separate experiments performed in duplicate (*$p < 0.05$; xx $p < 0.005$; + $p < 0.05$).

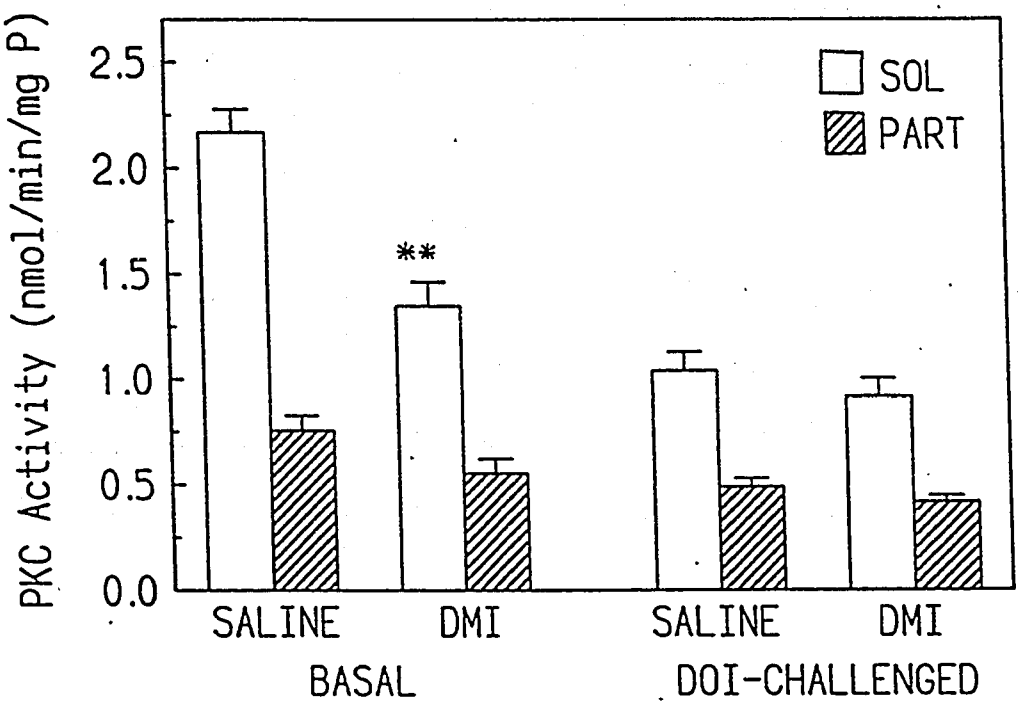

Fig. 26. Effect of chronic treatment with desipramine (10 mg/kg/day i.p. for 21 days) on basal and DOI-challenged (10 mg/kg i.p. one hour before death) PKC activity in soluble and particulate fractions of rat brain cortex compared with controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five separate experiments performed in duplicate ($^{**}p < 0.005$).

In contrast, basal PKC activity in both fractions of the hippocampus of desipramine-treated animals did not differ from controls (n = 5), whereas DOI pretreatment of animals (n = 5) resulted in a significant decrease in activity in the soluble fraction to 1.32 ± 0.13 nmol/min/mg protein compared to 1.78 ± 0.08 in saline-treated controls ($p < 0.05$) (Fig. 27). The distribution of activity in the hippocampus of DOI-challenged rats was 72.5 ± 1.65 % in the soluble and 27.5 ± 1.65 % in the particulate fractions.

The net response of desipramine-treated animals (n = 5) in response to the DOI challenge is presented in Figs. 28 (cortex) and 29 (hippocampus). Whereas saline-treated animals responded to acute DOI pretreatment with decreases in cortical soluble and particulate fraction PKC activities of 52.1 ± 4.14 % and 35.5 ± 5.56 % respectively, desipramine-treated rats responded to DOI with smaller decreases of activity (31.9 ± 6.48 % and 23.6 ± 5.5 % in cortical soluble and particulate fractions respectively) (Fig. 28). In the hippocampus, the response of saline-treated controls to DOI was a decrease in PKC activity in soluble and particulate fractions of 36.7 ± 2.93 % and 34.4 ± 5.78 %, respectively; desipramine-treated rats responded to the DOI challenge with a larger decrease in soluble PKC activity (46.8 ± 5.42 %) but with a smaller decrease in particulate activity (30.6 ± 5.98 %) (Fig. 29).

3.7 Effect of chronic fluoxetine treatment on PKC activity

Chronic treatment of rats (n = 5) with fluoxetine (5 mg/kg/day i.p. for 21 days) produced significant decreases in basal PKC activity in both fractions of the cortex;in the soluble

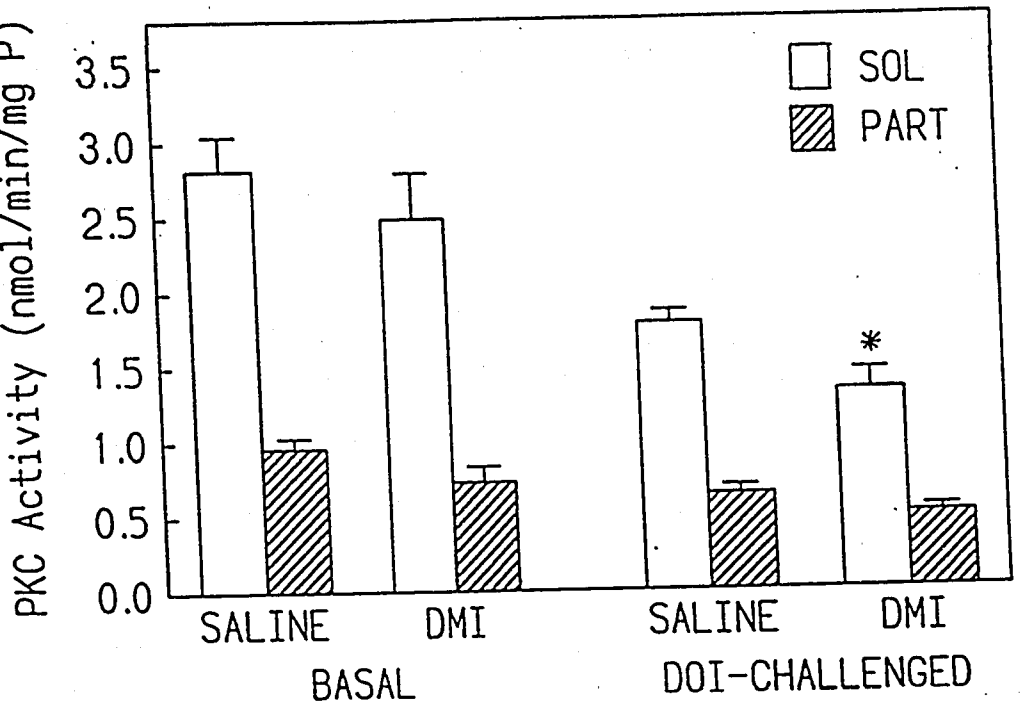

Fig. 27. Effect of chronic treatment with desipramine (10 mg/kg/day i.p. for 21 days) on basal and DOI-challenged (10 mg/kg i.p. one hour before death) PKC activity in soluble and particulate fractions of rat brain hippocampus compared with controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five separate experiments performed in duplicate ($^{*}p < 0.05$).

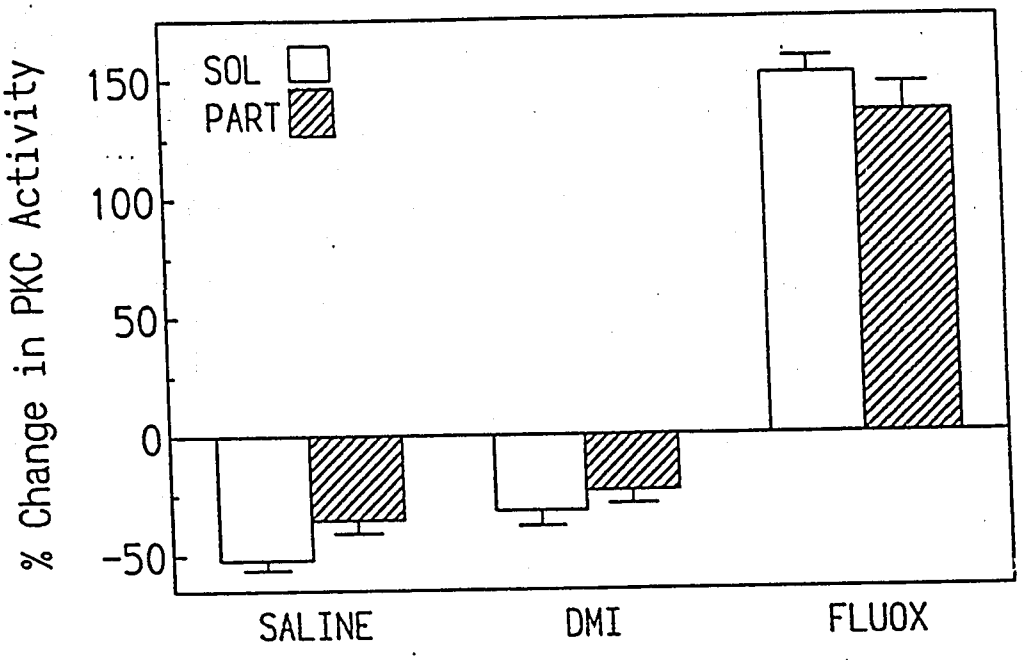

Fig. 28. Effect of chronic treatment with antidepressants (10 mg/kg desipramine or 5 mg/kg fluoxetine i.p. per day for 21 days) on % change in PKC activity in soluble and particulate fractions of rat brain cortex following one dose of DOI (10 mg/kg one hour before death) compared to controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five separate experiments performed in duplicate.

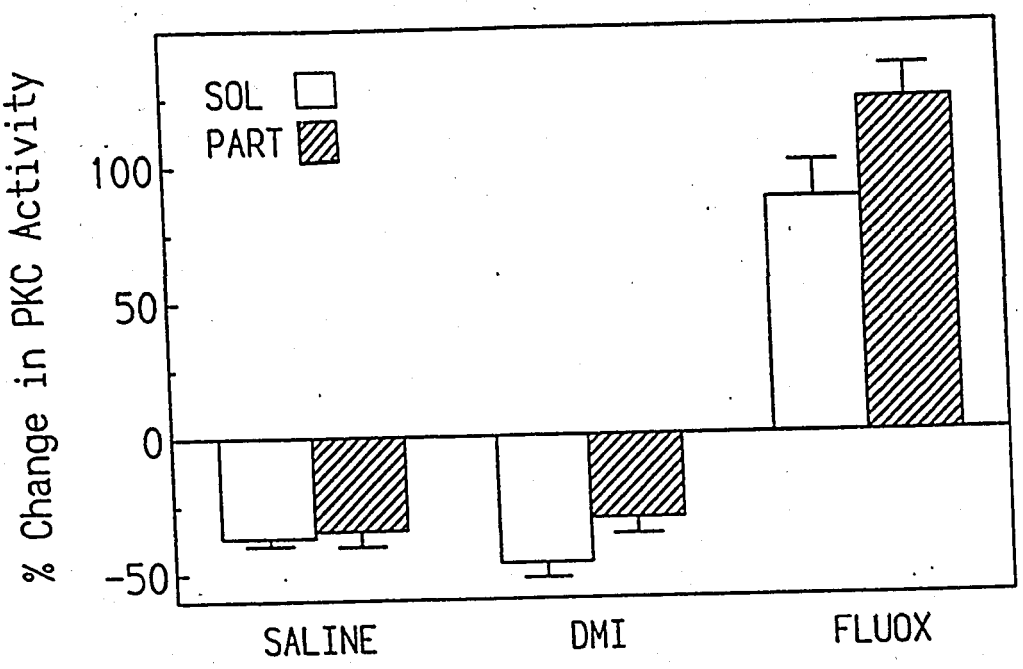

Fig. 29. Effect of chronic treatment with antidepressants (10 mg/kg desipramine or 5 mg/kg fluoxetine i.p. per day for 21 days) on % change in PKC activity in soluble and particulate fractions of rat brain hippocampus following one dose of DOI (10 mg/kg one hour before death) compared to controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five separate experiments performed in duplicate.

fraction from 2.17 ± 0.11 nmol/min/mg protein in saline-treated controls to 1.15 ± 0.03 in fluoxetine-treated animals and in the particulate fraction from 0.76 ± 0.07 nmol/min/mg protein in controls to 0.51 ± 0.02 nmol/min/mg protein in fluoxetine-treated animals ($p < 0.001$ in soluble and $p < 0.05$ in particulate) (Fig. 30). Distribution of PKC activity in fluoxetine-treated animals was 69.4 ± 0.80 % and 30.6 ± 0.80 % in soluble and particulate fractions, respectively. Activity in both cellular fractions of the cortex following challenge with DOI (10 mg/kg i.p. one hour before death) ($n = 4$) was dramatically increased in fluoxetine-treated animals in comparison with saline-treated controls. In the soluble fraction, the PKC activity was 2.89 ± 0.08 as compared to 1.04 ± 0.09 nmol/min/mg protein in control animals ($p < 0.001$), and in the particulate fraction, 1.20 ± 0.06 as compared to 0.49 ± 0.04 nmol/min/mg protein in control animals ($p < 0.001$). Relative distribution of PKC activity was 71.0 ± 0.85 % and 29.0 ± 0.85 % in soluble and particulate fractions, respectively.

Similarly, basal PKC activity in both fractions of the hippocampus of fluoxetine-treated animals was lower than that in controls ($n = 5$), with soluble activity decreasing to 1.95 ± 0.11 nmol/min/mg protein from 2.81 ± 0.23 nmol/min/mg protein in controls ($p < 0.05$). The PKC activity in the particulate fraction decreased to 0.66 ± 0.03 in fluoxetine-treated animals compared to 0.96 ± 0.07 nmol/min/mg protein in controls ($p < 0.05$). The relative distribution of activity in fluoxetine-treated animals was 74.5 ± 1.57 % and 25.5 ± 1.57 % in the soluble and particulate fractions, respectively (Fig. 31). As in the cortex, DOI-challenged PKC activity in the hippocampus of treated animals ($n = 4$) was increased from 1.78 ± 0.08 in controls to 3.64 ± 0.26 nmol/min/mg protein in fluoxetine-

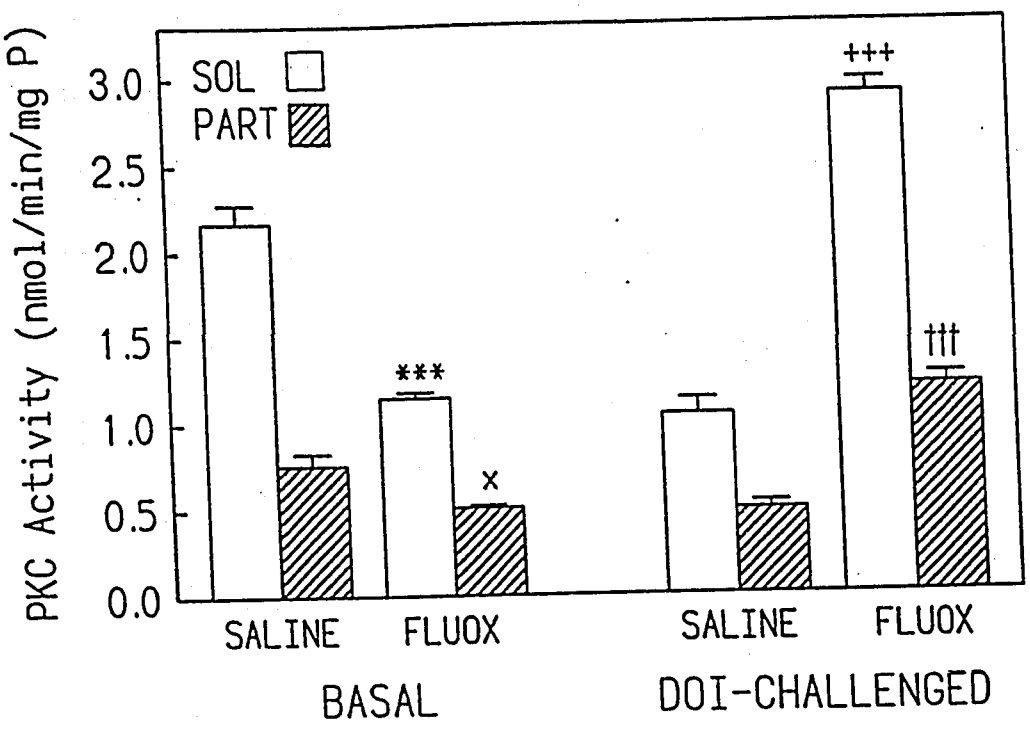

Fig. 30. Effect of chronic treatment with fluoxetine (5 mg/kg/day i.p. for 21 days) on basal and DOI-challenged (10 mg/kg i.p. one hour before death) PKC activity in soluble and particulate fractions of rat brain cortex compared with controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five (basal activity) or four (DOI-challenged activity) separate experiments performed in duplicate (***$p < 0.001$; x $p < 0.05$; +++$p < 0.001$; †††$p < 0.001$).

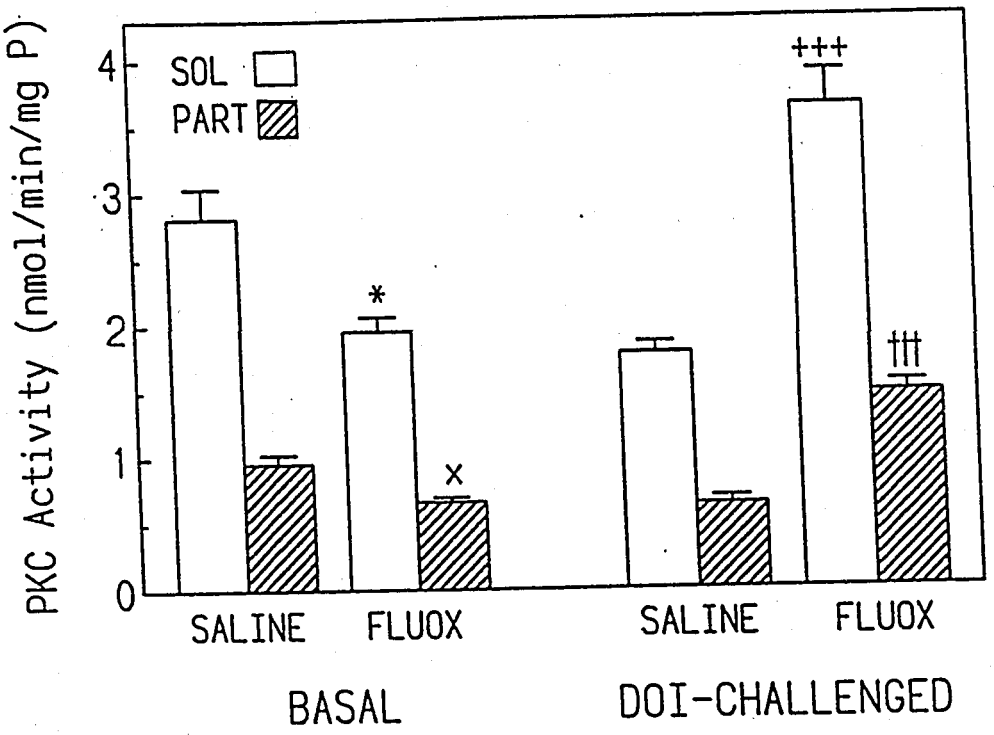

Fig. 31. Effect of chronic treatment with fluoxetine (5 mg/kg/day i.p. for 21 days) on basal and DOI-challenged (10 mg/kg i.p. one hour before death) PKC activity in soluble and particulate fractions of rat brain hippocampus compared with controls (equal volume of 0.9 % saline each day for 21 days). Incorporation of [^{32}P]ATP was measured at 3.3 μCi/mL [^{32}P]ATP. Non-specific PKC activity was defined by activity present in an equal volume of homogenate buffer alone. Each bar represents the mean ± SEM of five (basal activity) or four (DOI-challenged activity) separate experiments performed in duplicate (*$p < 0.05$; x $p < 0.05$; +++$p < 0.001$; †††$p < 0.001$).

treated animals in the soluble fraction ($p < 0.001$), and from 0.63 ± 0.06 in controls to 1.47 ± 0.08 nmol/min/mg protein in fluoxetine-treated animals in the particulate fraction ($p < 0.001$). The relative distribution of activity in treated animals was 71.5 ± 0.52 % and 28.5 ± 0.52 % in the soluble and particulate fractions, respectively.

The net response of fluoxetine-treated animals ($n = 5$) in response to the DOI challenge is presented in Figs. 28 (cortex) and 29 (hippocampus). Whereas saline-treated control animals responded to acute DOI pretreatment with decreases in cortical soluble and particulate fraction activities of 52.1 ± 4.14 % and 35.5 ± 5.56 % respectively, fluoxetine-treated rats responded to DOI with large increases in activity (151.3 ± 7.0 % and 135.3 ± 11.7 % in cortical soluble and particulate fractions respectively) (Fig. 28). In the hippocampus, the PKC activity in soluble and particulate fractions from saline-treated controls in response to DOI was decreased by 36.7 ± 2.93 % and 34.4 ± 5.78 %, respectively. The fluoxetine-treated response to the DOI challenge again was a large increase in PKC activity (86.7 ± 13.3 % and 122.7 ± 12.1 % in soluble and particulate fractions, respectively) (Fig.29).

IV. DISCUSSION

A. PRESYNAPTIC MECHANISMS: ANTIDEPRESSANTS AND SEROTONIN TRANSPORTER FUNCTION

1. Sodium dependence of [^{3}H]paroxetine binding and [^{3}H]5-HT uptake

The kinetic parameters of [^{3}H]paroxetine binding in rat diencephalon obtained in this study were close to those reported for rat brain (Habert et al, 1985; Laruelle et al, 1988; Edwards et al, 1991) or human brain (Laruelle et al, 1988), although other investigators have reported lower Kd values for human as well as rat brain (Marcusson et al, 1988; Lawrence et al, 1990). The higher Kd values in this experiment may have been due to a relatively large fraction of the radiolabel bound. Additional factors could be the differences in tissue preparation and assay conditions (amount of protein per tube, incubation volume, per cent nonspecific binding).

The sodium dependence of paroxetine binding had not been investigated before this study, although that of imipramine, a nonselective 5-HT uptake blocker, had been studied in both brain and platelets in terms of the nature of sodium requirement for binding. Talvenheimo et al (1983) found no change in the Bmax of [^{3}H]imipramine binding in platelets over increasing concentrations of sodium, although Kd increased significantly as sodium was reduced. In brain cerebrocortical membranes, Briley and Langer (1991) also found no change in the Bmax of [^{3}H]imipramine binding and a high Kd value in absence of NaCl. Both 1:1 and 1:2 ratios of imipramine molecules to sodium ions have been

suggested as requirements for [^{3}H]imipramine binding (Talvenheimo et al, 1983; Wood, 1987). Kinetic evidence based on the linear double reciprocal plot of [^{3}H]imipramine bound and NaCl concentration led Wood (1987) to suggest the 1:1 ratio, whereas Talvenheimo et al (1983) found a rectangular hyperbolic relationship between [^{3}H]imipramine affinity (1/Kd) and $[Na^+]^2$ suggesting that two sodium ions are required for binding of one imipramine molecule. However, the findings of sodium dependence of imipramine binding cannot simply be extended to the binding of paroxetine because paroxetine, in contrast to imipramine, appears to label a single class of high-affinity sites (Marcusson and Erikkson, 1988).

In the present study, the density of [^{3}H]paroxetine sites (Bmax) was independent of sodium concentration. This is similar to findings with [^{3}H]imipramine and some other ligands that label amine transporters, e.g., [^{3}H]mazindol (Zimanyi et al, 1989). In contrast, the affinity of [^{3}H]paroxetine for its binding sites was clearly sodium dependent. The kinetic analyses of the relationship between sodium concentration and the affinity of binding (1/Kd) or the amount of binding at single concentrations of ligand strongly suggest that one sodium ion is required for binding of the paroxetine molecule to the transporter. This is similar to the sodium requirement for high-affinity binding of [^{3}H]imipramine in brain tissue (Wood, 1987), and for [^{3}H]5-HT binding and translocation in brain and platelets (Talvenheimo et al,1983; Wood, 1987; present data). The results thus further support the single-site model of the neuronal 5-HT uptake and imipramine-binding site originally suggested by Marcusson et al (1986). The sodium dependence of Vmax of [^{3}H]5-HT uptake, in contrast to Bmax of [^{3}H]paroxetine binding, probably reflects

the sodium requirement of the translocation step that follows the binding of 5-HT to its recognition site.

The Vmax of [^{3}H]5-HT uptake in platelets was found to be clearly sodium dependent at ≤ 200 mM NaCl (Talvenheimo et al, 1983); a similar sodium dependence of Vmax in rat diencephalon is now reported. The sodium dependence of the Km of 5-HT uptake has been controversial in past reports. Km was demonstrated to be significantly sodium dependent in human and rat platelets (Sneddon, 1969; Talvenheimo et al, 1983) and in guinea-pig striatal tissue (Kilpatrick et al, 1986). In agreement with these findings, the Km of [^{3}H]5-HT uptake in this experiment was found to be significantly sodium dependent in the rat diencephalon. In contrast, Wood (1987) reported no sodium dependence of the Km of [^{3}H]5-HT uptake in rat brain cortex. However, his kinetic analysis of the effect of sodium on the uptake of 100 nM [^{3}H]5-HT yielded a linear regression line indicating that the transport of one 5-HT molecule was associated with the interaction of one sodium ion. A similar linear regression using 50 nM [^{3}H]5-HT concentration was obtained in this experiment. The hyperbolic relationship between external [Na^+] and both uptake affinity (1/Km) and rate (Vmax) suggests that one sodium ion is required for both binding and subsequent translocation of serotonin. Recently, O'Reilly and Reith (1988) suggested that in membrane vesicles from mouse cerebral cortex, two sodium ions are required for serotonin uptake. This finding is at variance with the sodium requirement for 5-HT uptake in rat diencephalon (present study) and human platelets (Talvenheimo et al, 1983). The reason for this discrepancy may be in the preparations, tissues, or species examined. Direct estimation of the number of sodium ions actually cotransported together with

serotonin may have to be used to resolve this discrepancy. The recent cloning and expression of a functional serotonin transporter from rat brain (Blakely et al, 1991; Hoffman et al, 1991) may in the near future facilitate further studies to elucidate the nature of 5-HT transport and of the molecular pharmacology of this transporter.

In conclusion, [^{3}H]paroxetine binding and [^{3}H]5-HT uptake in brain diencephalon have similar requirements for external sodium. The binding of [^{3}H]paroxetine as well as the binding and subsequent translocation of [^{3}H]5-HT both require 1:1 ratios to sodium ions. This finding concurs with a single-site model of the sodium-dependent serotonin neuronal transporter with common or overlapping domains on the substrate recognition site that would bind serotonin and the tricyclic and nontricyclic 5-HT uptake inhibitors.

2. Effect of chronic antidepressant treatment on [^{3}H]5-HT uptake

Chronic treatment with desipramine failed to alter the kinetic parameters of 5-HT uptake in either the cortex or the hippocampus. Desipramine has previously been shown to have greater affinity for the noradrenaline transporter than for the 5-HT transporter, and to block the uptake of noradrenaline more efficiently than that of 5-HT (Ross and Renyi, 1975). Although secondary tricyclic antidepressants, desipramine and nortriptyline inhibit 5-HT uptake (Ross and Renyi, 1975), chronic administration of nortriptyline was shown not to produce changes in 5-HT uptake parameters (Hrdina, 1987). Therefore, it is not surprising that chronic treatment with desipramine in the present experiments altered neither the rate nor the affinity of uptake of 5-HT.

Conversely, chronic treatment with fluoxetine resulted in a significant decrease in Vmax and increase in Km of 5-HT uptake. Fluoxetine is a potent blocker of 5-HT uptake which binds with high affinity and selectivity to the serotonin transporter (Wong et al, 1983), and thus the decrease in rate of 5-HT uptake was expected. In fact, chronic administration of fluoxetine was reported to significantly decrease 5-HT uptake in rat cortex (Hrdina, 1987; Caccia et al, 1992) and to produce a significant reduction in amount of 5-HT and of 5-HIAA. The decrease in 5-HT reported (Hrdina, 1987) could have resulted from the persistent blockage of 5-HT uptake by fluoxetine in presence of continuous use of the transporter to uphold serotonergic transmission. However, the increase in Km (decrease in affinity) after treatment in the present experiments was not anticipated; the antagonism by fluoxetine of 5-HT binding to the substrate recognition site is likely of a competitive nature, if fluoxetine labels the 5-HT recognition site itself (Hrdina, 1988). However, it may be postulated that while fluoxetine directly blocks 5-HT uptake, the changes in uptake parameters following chronic treatment need not necessarily be related to the immediate uptake-blocking action of the drug. Chronic treatment with imipramine, for example, does not result in any changes in parameters of 5-HT uptake regardless of its efficiency as a 5-HT uptake blocker (Barbaccia et al, 1983). Thus it is reasonable to assume that fluoxetine may also interact in the long-term with some aspect of the presynaptic machinery of the serotonergic synapse, resulting ultimately in less efficacious uptake of serotonin by the transporter (discussed below in "Effects of antidepressants on PKC activity"). However, an effect of the residual drug on 5-HT uptake parameters also has to be considered since fluoxetine (and its active metabolite norfluoxetine) were shown to significantly accumulate in cerebral cortex and hippocampus after repeated administation

(Caccia et al, 1992).

B. POSTRECEPTOR MECHANISMS: ANTIDEPRESSANTS AND PKC

1. Effect of antidepressants on PKC location

The location of PKC in rat brain was quantitated by determining the [^{3}H]PDBu binding in both brain sections and homogenates. The amount of PKC distributed in the soluble and particulate fractions of the cortical and hippocampal homogenates was higher than that reported previously in human brain (Horsburgh et al, 1991), although the difference in amount may reflect the difference in species used. The amount of PKC in brain sections was also higher than that reported previously (Hara et al, 1991; Kawagoe et al, 1991). In the present experiment, [^{3}H]PDBu binding at 2.5 nM [^{3}H]PDBu was about twice as high in brain sections as in particulate fractions of homogenates, and may reflect the higher per cent specific binding obtained in sections (95 %) as opposed to that obtained in particulate fractions of homogenates (80 to 90 %). In addition, the amount of protein per brain section is less accurately measured than that in an aliquot of homogenate, and this could affect the amount of [^{3}H]PDBu bound when expressed in pmol/mg protein.

The overall trend towards lower binding of [^{3}H]PDBu in all brain areas (significant in layers 4 to 6 of parietal cortex and striatum) following acute treatment with desipramine indicates lower binding to membranes is in agreement with the data presented for PKC activity in subcelllular fractions following acute desipramine treatment, and is further discussed in

that section.

The decrease in the amount of PKC in the soluble fraction of the cortex following one dose of the 5-HT_2 agonist DOI was expected because it has been established that PKC translocates acutely from the cytosol to the membrane following stimulation of receptors that are linked to the phosphoinositide cascade (Nishizuka, 1986), including the 5-HT_2 receptor (Roth et al, 1986; Dillon-Carter and Chuang, 1989; Kagaya et al, 1990). In particular, DOI itself has been used as a 5-HT_2 agonist *in vitro* to demonstrate PKC activity translocation to the membrane following 5-HT_2 receptor activation (Wang and Friedman, 1990). The decrease in amount of PKC in the particulate fraction observed in this experiment following acute treatment with DOI was not expected. However, this is the first report of changes in PKC location following the *in vivo* administration of DOI and thus it is possible that in the hour between administration of the drug and the measurement of PKC location, potential translocation of PKC to the membrane could have terminated while the decrease in cytosolic PKC could persist for a longer time period. In the hippocampus, there was also a trend towards a decrease in amount of PKC associated with the cytosol although it did not reach statistical significance. The less obvious decrease in hippocampal cytosolic PKC following DOI administration may reflect the fact that the density of DOI binding (and hence the density of 5-HT_2 receptors) is low in the hippocampus as compared to the cortex (Appel et al, 1991), and hence there are less opportunities for DOI to elicit the translocation of PKC through binding to and activation of 5-HT_2 receptors.

Gleiter et al (1988) has reported a decrease in amount of cortical and cerebellar membrane-bound PKC following chronic but not acute treatment with electroconvulsive shock therapy (decrease in Bmax of [^{3}H]PDBu binding). There was no change in the amount of PKC associated with either cellular fraction of either tissue following chronic treatment with desipramine in the present experiment. However, this does not rule out the possibility that there may be a change in PKC location after 5-HT_2 receptor stimulation in animals treated chronically with desipramine. Or, potential changes in PKC after chronic antidepressant treatment may not be reflected in the enzyme location but rather in its activity, ie. the basal activity of the enzyme could change in a cellular fraction without there necessarily being any change in the number of enzyme molecules present.

2. Effect of antidepressants on PKC activity

The control values obtained in this experiment for PKC activity in the soluble and particulate fractions of the cortex and the hippocampus were similar to previous reports. (Wang and Friedman, 1990; Daigen et al, 1991). Interestingly, activity in the hippocampus showed a trend, though not statistically significant, towards higher values than in the cortex. The subcellular distribution of PKC activity was about 75 % in the soluble fraction and about 25 % in the particulate fraction, a distribution consistently reported in previous studies (Wang and Friedman, 1990; Daigen et al, 1991). It was found that the concentration of protein per tube was extremely crucial in determining the PKC activity. The optimal protein concentration per tube was low (less than 10 μg per tube) and any deviation from this concentration produced significant changes in PKC activity. It may be

that tubes containing higher than optimal protein concentrations have lower PKC activity values due to the presence of endogenous PKC inhibitors.

In agreement with the data for PKC location, there was a significant decrease in activity in both cellular fractions of the cortex following a single dose of DOI. The phenomenon of a decrease in cytosolic PKC activity induced by DOI has been previously demonstrated *in vitro* (Wang and Friedman, 1990), but this is the first report of such a decrease after *in vivo* exposure to the drug. That there was no translocation of PKC to the membrane (decrease also in particulate fraction PKC activity) may be due to the one hour time period between administration of the drug and sacrifice of animals, in that potential translocation of PKC to the membrane could have terminated in this time period while decrease in cytosolic PKC activity could persist for a longer time period. Treatment of animals with a single dose of desipramine resulted in decreases in particulate but not cytosolic fraction PKC activity in both tissues, and may be due to a direct interaction of the drug with the 5-HT_2 receptor-transducing system.

The tricyclic antidepressant drugs have been traditionally thought of as acting extracellularly in the synapse by blocking amine uptake. However, recent research has focused on elucidating potential intracellular actions of these drugs. Nalepa and Vetulani (1991) have shown that brief *in vitro* exposure of rat cortical slices to imipramine results in two effects. Firstly, imipramine acutely antagonizes α_1 receptors which are coupled to the phosphoinositide cascade, as evidenced by the inhibition of noradrenaline-stimulated inositol phosphate formation. Secondly, imipramine actually inhibits PKC, as evidenced

by its inhibition of the PKC potentiation of cAMP accumulation in response to ß-adrenoceptor agonists, as well as its inhibition of the PKC inhibition of inositol phosphate formation in response to α_1 agonists. These two acute effects of imipramine (direct α_1 receptor antagonism and PKC inhibition) in combination would ultimately reduce the response of ß-adrenoceptors to agonists, as the stimulation of α_1 receptors is known to potentiate ß-mediated cAMP accumulation (Daly et al, 1980; Duman and Enna, 1987). It is possible that acute *in vivo* desipramine may behave in a manner similar to that of *in vitro* imipramine described above, in terms of inhibiting PKC. Why the decrease in PKC activity in this experiment was only evident in the membrane fraction remains to be established.

Recently there have been reports that *in vitro* exposure of platelets as well as cortical tissue to desipramine leads to inhibition of phosphoinositide turnover (Pandey et al, 1991[a]; Pandey et al, 1991[b]). In platelets thrombin-induced IP_2 and IP_3 formation was inhibited by preincubation with desipramine and imipramine (Pandey et al, 1991[a]); in brain tissue, the same preincubation resulted in decreased 5-HT-stimulated IP_1 formation (Pandey et al, 1991[b]). It was suggested that the inhibition of phosphoinositide turnover was due to a direct and acute inhibition of PLC by desipramine. Therefore, another possible explanation for the decrease in membrane PKC activity following acute *in vivo* administration of desipramine could be inhibition of PLC resulting in decreased PKC activity in the membrane fraction.

Treatment of animals with a single dose of fluoxetine resulted in the same pattern of

changes in PKC activity to those achieved with DOI (decrease in activity in both fractions of cortex, decrease only in particulate fraction of hippocampus). One possible explanation is that, subsequent to the blockage of uptake of serotonin which occurs soon after the administration of fluoxetine (Wong et al, 1985), the increased amount of serotonin in the synapse would then be available to act on 5-HT_2 receptors and hence to elicit the translocation of PKC to the membrane that is known to occur following the stimulation of this receptor with agonist. It is difficult to explain why there was a decrease in particulate fraction PKC activity; if indeed acute treatment with fluoxetine mirrors the effect of acute treatment with DOI due to increased stimulation of 5-HT_2 receptors with endogenous 5-HT, the decrease in particulate fraction PKC activity may again be explained by the time lapse between administration of the drug and sacrifice of animals (as discussed for both effect of acute DOI on PKC location and effect of acute DOI on PKC activity). Again, the lack of decrease of hippocampal cytosolic PKC activity probably reflects the smaller number of 5-HT_2 receptors in this tissue (Pazos et al, 1985).

Chronic treatment with desipramine resulted in a change similar to that observed by Nestler et al (1989) in PKA activity following chronic imipramine treatment. In both cases, chronic treatment with the drug resulted in a significant decrease in the basal activity of PKA (imipramine, Nestler et al, 1989) or PKC (desipramine, present study) associated with the cytosolic fraction of the cortex. That there was no increase in particulate PKC activity could be a reflection of a long-term translocation of activity to the nucleus, which was found to be the case with PKA after long-term imipramine therapy (Nestler et al, 1989). The consequence of a movement of PKC to the nucleus during the course of

antidepressant treatment is unclear. It has been shown that protein kinases can regulate gene expression via actions on DNA regulatory elements. One possible target for change in gene expression is G-proteins. The 5-HT_2 and 5-HT_{1A} receptors are coupled to PLC and adenylate cyclase by G_o and G_i proteins, respectively. Lesch et al (1991) have quantitated the α subunits of G proteins following chronic tricyclic antidepressant treatment. They reported an overall brain decrease in G_s and G_i proteins but an overall increase in G_o proteins, with the increase in G_o protein much greater in the cortex (70 % increase) than in the hippocampus (20 % increase). It was postulated that the previously-established 5-HT_2 receptor downregulation following chronic antidepressant treatment (Peroutka and Snyder, 1980) may well be overridden by this large increase in amount of G_o protein, resulting in enhanced efficacy of the 5-HT_2 receptor-transducing system, and that the decrease in brain G_i protein may explain the subsensitivity of 5-HT_{1A} receptor signalling reported from electrophysiological studies (Blier et al, 1990). Therefore, a plausible role for translocation of PKC activity to the nucleus following chronic desipramine treatment, that of differential G-protein gene expression, exists.

Interestingly, although there was no corresponding change in basal cytosolic activity in the hippocampus, there was a significant decrease in the activity in response to DOI stimulation. Hence serotonergic transmission (in terms of PKC activation) in the hippocampus was altered by desipramine treatment on a functional level rather than on a basal level, perhaps by sensitization of the receptor to agonist. However, 5-HT_2 receptor number is down-regulated by chronic desipramine treatment (Peroutka and Snyder, 1980), and therefore the increased response of PKC to DOI in the hippocampus cannot

likely be explained by a concomitant increase in 5-HT_2 receptors. In fact, while cytosolic PKC activity in saline-treated animals decreased by 52 % after DOI challenge, activity in desipramine-treated animals decreased by only 32 % in the cortex (reflecting a change in basal enzymatic activity unaccompanied by a change in translocating ability of PKC) but in the hippocampus activity decreased by 47 % as compared to controls where it only decreased by 37 % (reflecting a change in translocating ability of PKC unaccompanied by a change in basal enzymatic activity).

Decreases in basal PKC activity in the cytosol of both tissues following chronic fluoxetine treatment may be due to a permanent movement of PKC away from the cytosol to another area of the cell, perhaps to the nucleus, as suggested by Nestler et al (1989) in the case of PKA after chronic imipramine treatment. Although the decrease in cytosolic activity was also observed after acute treatment with the drug, the reasons for the decreases after acute and chronic treatment are probably not the same because enough time (48 hours) elapsed between the last daily dose of the drug and the assay of PKC activity that one can rule out immediate effects of the drug. However, the persistent effect of residual drug cannot be excluded (Caccia et al, 1992). The large increases in PKC activity in both fractions of both tissues following DOI challenge (151 % and 135% in the soluble and particulate fractions and 87 and 123% in the soluble and particulate fractions of the cortex and hippocampus, respectively) in fluoxetine-treated rats was not expected and contrasted the results obtained in desipramine-treated rats. One possibility is that this effect may be due to a change in PKC activity in the presynaptic terminals following chronic treatment. A review of presynaptic serotonin transporter regulation is thus

warranted.

It appears from recent evidence that the activity of the presynaptic 5-HT transporter is regulated by at least one presynaptic receptor. This receptor may or may not be the putative 5-HT_{1B} autoreceptor. Cool et al (1991) have used the JAR human choriocarcinoma cell line which expresses a functional serotonin transporter as a model for studying serotonin transporters in general. It was found that cholera toxin, which ADP-ribosylates the α subunit of G proteins and results in a continuous generation of cAMP, increased the Vmax of serotonin uptake by 122 %. The stimulatory effect of cholera on 5-HT uptake was antagonized by a protein kinase inhibitor. This study suggests that the serotonin transporter is activated by a cAMP-dependent protein kinase (PKA).

Recent evidence has shown that PKC is also involved presynaptically in the uptake of 5-HT, in this case in the inhibition of uptake. In basophilic leukemia cells, Hoffman (1991) reported a 53 % decrease in [^{3}H]5-HT uptake following 16 hour preincubation of cells in the presence of a PKC activator. In the same experiment, culture of cells in 10 μM cAMP analogue resulted in a 225 % increase in [^{3}H]5-HT uptake. The 5-HT presynaptic autoreceptor, postulated to be the 5-HT_{1B} receptor linked to the adenylate cyclase cascade, is responsible for the negative feedback inhibition on 5-HT release (Engel et al, 1983). It is possible that stimulation of the 5-HT_{1B} receptor results in an increase in intracellular cAMP which then serves to both inhibit release of serotonin and also to stimulate uptake of serotonin, the final result being an overall decrease in serotonergic transmission. PKC activation has been shown to stimulate release of neurotransmitters

(Huang et al, 1988; Dekker et al, 1991). The finding of a decrease in serotonin uptake induced by PKC activator in the Hoffman experiment raises the possibility that the activity of the transporter is linked to one or more receptors via one or more second messenger cascades. One messenger, cAMP, whether or not linked to the autoreceptor, would increase activity of the transporter while PKC would decrease its activity, as necessary. Although the autoreceptor has only been shown to be linked to the adenylate cyclase cascade, it appears that at least one other presynaptic receptor type, hypothetically linked to the phosphoinositide pathway, partially regulates transporter function via an action by PKC. In agreement with the Hoffman study, a second study (Anderson et al, 1991) has shown that PKC activator decreases the Vmax of 5-HT uptake by 34 % in platelets. No change in [^{3}H]imipramine binding was found after treatment and thus decrease in transporter number was not an explanation for the inhibitory effect of PKC activator on 5-HT uptake. Therefore, PKC appears to down-regulate the activity of the transporter, and the results of this study lend further support to the notion of a presynaptic receptor linked to PKC which, upon stimulation, would cause a decrease in serotonin uptake mediated by PKC.

DOI is a potent ligand at 5-HT_2 and 5-HT_{1C} receptors. 5-HT_{1C} receptors are most abundant in the choroid plexus but are also present in cortex and hippocampus. In experimental brain membrane preparations, it is difficult to differentiate between presynaptic and postsynaptic receptors as these two areas of the neuron are not easily separated. Electrophysiological studies suggest that 5-HT_2 receptors are postsynaptic, but the possibility that they or another heretofore unknown receptor also stimulated by

DOI (5-HT_{1c} or otherwise) may be situated presynaptically as well cannot be ruled out. Future experiments in rats with lesions to serotonergic terminals could shed more light on this issue. The dramatic increases in PKC activity following DOI challenge in chronic fluoxetine-treated rats observed in this experiment may be occurring presynaptically. The large increase in PKC activity upon DOI challenge may explain why the Vmax of [^{3}H]5-HT uptake was decreased following long-term treatment with the drug, if indeed transporter function is inhibited in neurons by PKC activation as it is in basophilic leukemia cells (Hoffman, 1991) and in platelets (Anderson et al, 1991). The mechanism by which fluoxetine would change the response of the cell to DOI in terms of increasing the activity of PKC while not increasing, but rather decreasing, the basal activity of the cell is unclear and will have to be further investigated. It is of interest that chronic desipramine treatment caused no such increase in PKC activity (either basal or DOI-challenged) and neither did it result in any change in kinetic parameters of [^{3}H]5-HT uptake.

In conclusion, it appears that antidepressant drugs produce significant changes in PKC activity in the subcellular fractions of serotonergic neurons both after acute and chronic treatment. These changes may be significant in the terms of altering transduction of cellular signals evoked by binding of 5-HT to receptors.

Future experiments will have to look at changes in nuclear fraction PKC activity following long term administration of antidepressants in order to evaluate potential regulation of gene expression mediated by PKC or PKC-activated phosphoproteins. When evaluating the response of tissue subjected to chronic antidepressant treatment to DOI stimulation,

it will be important to assess the effect of blockage of 5-HT_2 receptors prior to DOI administration in order to confirm that changes in PKC activity following the DOI challenge are indeed due to stimulation of the 5-HT_2 receptor.

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Sodium Dependence of [^{3}H]Paroxetine Binding and 5-[^{3}H]Hydroxytryptamine Uptake in Rat Diencephalon

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Abstract: The sodium dependence of binding of [^{3}H]-paroxetine, a selective serotonin uptake inhibitor, to the serotonin transporter in rat diencephalon was studied in both brain membranes and tissue sections and compared with that of 5-[^{3}H]hydroxytryptamine ([^{3}H]5-HT) uptake by synaptosomes from the same region. Binding of [^{3}H]-paroxetine in both the membranes and sections displayed clear sodium dependence until a plateau occurring at 60 m*M* NaCl, the EC_{50} for sodium being 8 and 25 m*M*, respectively. The affinity ($1/K_D$) of [^{3}H]paroxetine binding was a simple hyperbolic function of sodium concentration. In contrast, the density of [^{3}H]paroxetine sites was not affected by external Na^+ concentration. The uptake of [^{3}H]5-HT showed a similar pattern of sodium dependence with an EC_{50} for Na^+ of 25 m*M*. Both the affinity ($1/K_m$) and the rate (V_{max}) of [^{3}H]5-HT uptake were dependent on external [Na^+] with sodium-dependence curves fitting a rectangular hyperbola. The kinetic analysis of results indicates that one sodium ion is required for the binding of [^{3}H]paroxetine as well as for the binding and translocation of each [^{3}H]5-HT molecule. The results concur with a single-site model of the sodium-dependent serotonin transporter with common or overlapping domains for 5-HT and 5-HT uptake inhibitors.
Key Words: Paroxetine binding—5-Hydroxytryptamine uptake—Sodium dependence—Rat diencephalon. **Mann C. D. and Hrdina P. D.** Sodium dependence of [^{3}H]-paroxetine binding and 5-[^{3}H]hydroxytryptamine uptake in rat diencephalon. *J. Neurochem.* **59**, 1856–1861 (1992).

The high-affinity neuronal uptake of 5-hydroxytryptamine (5-HT) is an important carrier-mediated mechanism for the removal of this amine from the synaptic cleft. It has been demonstrated to be a sodium-dependent process in the brain and spinal cord of various species (Blackburn et al., 1967; Stauderman and Jones, 1985; Kilpatrick et al., 1986; Wood, 1987; O'Reilly, 1988). The uptake process involves several steps; after binding of the 5-HT molecule to the substrate recognition site, the molecule is translocated by a carrier to the inside of the cell where it then dissociates from the carrier. Certain drugs, including the tricyclic antidepressants, are known to inhibit the neuronal uptake of 5-HT, thus increasing the amount of transmitter in the synapse and hence its action on both pre- and postsynaptic receptors.

[^{3}H]Imipramine has been shown to bind with high affinity to the 5-HT transporter complex in both serotonergic neurons and platelets (Briley et al., 1979; Raisman et al., 1979). It was originally thought to act by allosterically regulating the function of the transporter although not itself binding to the substrate recognition site (Langer et al., 1980; Langer and Raisman, 1983). Imipramine, however, binds in brain tissue to two classes of sites, high and low affinity (Hrdina, 1984; Conway and Brunswick, 1985; Moret and Briley, 1986; Marcusson et al., 1986); only the high-affinity site has been found to be functionally related to the 5-HT uptake process (Marcusson et al., 1986; Hrdina, 1988). Recent studies using a more selective 5-HT uptake blocker, paroxetine, have shown that drugs affecting 5-HT uptake likely bind to the substrate recognition site itself (Habert et al., 1985; Humphreys et al., 1988; Marcusson and Eriksson, 1988; Hrdina et al., 1990). Although [^{3}H]imipramine binding has been found to be a sodium-dependent process (Briley and Langer, 1981), the ratio of imipramine molecules to sodium ions involved in the imipramine binding process has been controversial. It was suggested (Talvenheimo et al., 1983) that in platelets,

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Abbreviations used: ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine or serotonin.

two sodium ions are required for the binding of one imipramine molecule; however, in rat brain cortex (Wood, 1987), a 1:1 ratio based on kinetic evidence was suggested. The kinetics of the sodium dependence of [^{3}H]paroxetine binding have not yet been studied.

The sodium dependence of 5-HT uptake has been clearly demonstrated. However, several reports are at variance as to the nature of sodium requirement for the binding and translocation steps involved in serotonin transport (Talvenheimo et al., 1983; Stauderman and Jones, 1985; Kilpatrick et al., 1986; Wood, 1987; O'Reilly and Reith, 1988). [^{3}H]Paroxetine has recently been shown to be a suitable marker not only of the serotonin transporter, but also of serotonergic innervation in the brain (Hrdina et al., 1990; Dewar et al., 1991). We have thus investigated the significance of sodium ions for [^{3}H]paroxetine binding to the 5-HT transporter in both membranes and sections of rat diencephalon that includes many structures rich in serotonergic innervation (Parent et al., 1981; Hrdina et al., 1990), and have compared it with the sodium dependence of [^{3}H]5-HT uptake kinetics in synaptosomes from the same part of the brain.

MATERIALS AND METHODS

Materials

[^{3}H]5-HT creatinine sulfate (sp. act. 23.2 Ci/mmol) and [^{3}H]paroxetine (sp. act. 25 Ci/mmol) were purchased from NEN Chemicals (Boston, MA, U.S.A.). Fluoxetine-HCl was generously donated by Eli Lilly and Co. (Indianapolis, IN, U.S.A.), and pargyline-HCl by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were of purest grade available.

[^{3}H]Paroxetine binding

Binding of [^{3}H]paroxetine in membrane preparations from rat diencephalon was determined as described by Habert et al. (1985). In brief, male Sprague–Dawley rats (150–175 g) were killed by decapitation, brains dissected out and quickly removed. A coronal segment of diencephalon (from 3 to 7 mm anterior to the interaural line) was homogenized in 200 volumes of incubation buffer (50 m*M* Tris, 5 m*M* KCl, and concentrations of NaCl ranging from 0 to 200 m*M*, pH 7.4) for 15 s with a Polytron (setting 6). Four animals were used in separate trials at each of six different NaCl concentrations. The homogenate was centrifuged at 30,000 *g* for 10 min; the pellet obtained was resuspended in the original volume of incubation buffer, homogenized, and centrifuged again. The final pellet was resuspended in the original volume of buffer and kept on ice. To each tube containing 550 μl of incubation buffer (in the presence or absence of 10 μ*M* fluoxetine, for determination of total and nonspecific binding, respectively) was added 1,000 μl of homogenate, and 50 μl of [^{3}H]paroxetine in concentrations ranging from 0.05 to 2.0 n*M*. The final concentration of protein in incubation tubes was between 350 and 400 μg. Tubes were incubated in triplicate for 1 h at room temperature (20–22°C) after which incubation was terminated by addition of 5 ml of ice-cold incubation buffer containing 0.1% polyethyleneimine and subsequent filtration through Whatman GF/B filters followed by two additional washes. The filters were dried and the retained radioactivity was determined by liquid scintillation spectrometry. The maximum number of binding sites (B_{max}) and equilibrium dissociation constant (K_D) were calculated by Scatchard analysis of saturation binding data. The fraction of specific bound dpm versus total dpm added ranged between 4 and 10% and that of total bound dpm versus total dpm added between 8 and 16% depending on the concentration of the ligand. The effect of NaCl on [^{3}H]paroxetine binding was assessed without equiosmotic replacement of Na^+ in the incubation buffer because preliminary experiments have shown that [^{3}H]paroxetine binding at lower NaCl concentration without Na^+ replacement was not significantly different from that seen in a buffer with Na^+ equiosmotically replaced by Tris-HCl (data not shown).

For determination of [^{3}H]paroxetine binding in brain sections, rats were anesthetized with sodium pentobarbital and perfused via the left heart ventricle with Krebs–phosphate buffer and 0.1% formaldehyde. Brains were removed and stored at −60°C. Coronal sections of 10-μm thickness cut from four brains 6–7 mm anterior to the interaural line were processed as described earlier (Hrdina et al., 1990) and used for binding experiments. All sections were preincubated at room temperature (20–22°C) for 15 min in 50 m*M* Tris buffer. Incubation in duplicate proceeded at room temperature for 60 min in buffers containing [^{3}H]paroxetine (0.4 n*M*) and concentrations of NaCl ranging from 0 to 300 m*M*. To estimate the nonspecific binding, adjacent sections were incubated in the presence of 30 μ*M* fluoxetine. After incubation, the sections were washed twice for 20 min, and then wiped off the slides for liquid scintillation counting.

The specific binding of [^{3}H]paroxetine at concentrations of [^{3}H]paroxetine approximating its K_D (0.25 n*M* for membranes, 0.4 n*M* for brain sections) amounted to between 60 and 90% of total binding.

[^{3}H]5-HT uptake

The uptake of [^{3}H]5-HT by brain synaptosomal (P_2) preparation was determined as described earlier (Hrdina, 1987). Male Sprague–Dawley rats (150–175 g) were killed by decapitation and their brains dissected out and quickly removed. A coronal segment of diencephalon (from 3 to 7 mm anterior to the interaural line) was homogenized in 10 volumes of sucrose buffer (0.32 *M* sucrose, 0.002 *M* Tris, pH 7.4) with a Teflon pestle homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min and the resulting supernatant centrifuged at 17,000 *g* for 20 min. The final pellet was resuspended in the original volume of buffer and kept on ice. Fifty microliters of homogenate was added to tubes containing 400 μl of incubation buffer (20 m*M* Tris, 5 m*M* KCl, 1.2 m*M* $MgCl_2 \cdot 6H_2O$, 2.5 m*M* $CaCl_2 \cdot 2H_2O$, 10 m*M* glucose, 0.1 m*M* pargyline, 1 m*M* ascorbic acid, and concentrations of NaCl ranging from 0 to 120 m*M*, pH 7.4) aerated with 95% O_2 and 5% CO_2. Three animals were used in separate trials at each of five different NaCl concentrations. We found in preliminary experiments that [^{3}H]5-HT uptake at lower NaCl concentrations (<120 m*M*) without Na^+ replacement was significantly reduced in comparison with uptake measured in an incubation buffer with Na^+ equiosmotically replaced by LiCl (data not shown). It has been demonstrated earlier (Kilpatrick et al., 1986) that lithium possessed no intrinsic activity toward the uptake pump for 5-HT, indicating the suitability of this ion for replacement of sodium in serotonin uptake assays (Wood, 1987; O'Reilly and Reith, 1988). In contrast, a reduced [^{3}H]5-HT accumulation has been reported to occur when Na^+ was replaced by

$Tris^+$ in the incubation medium (Stauderman and Jones, 1985). To assess the effect of lower sodium concentrations (<120 m*M*) on serotonin uptake and maintain the osmolality of the buffer, NaCl was therefore replaced by equiosmolar amounts of LiCl. The tubes were preincubated at 37°C for 7 min; then 50 μl of [^{3}H]5-HT in concentrations ranging from 40 to 100 n*M* was added to each tube and a 2-min incubation in triplicate followed. Blank tubes representing nonspecific uptake were incubated at 0°C. Incubation was terminated by the addition of 5 ml of ice-cold incubation buffer and subsequent filtration through Whatman GF/B filters. The filters were washed twice with 5 ml of ice-cold incubation buffer, dried, and the retained radioactivity determined by liquid scintillation spectrometry. Net uptake was defined as the difference between the total (37°C incubation) and the nonspecific (0°C incubation) uptake. The maximum uptake rate (V_{max}) and affinity constant (K_m) were calculated from Lineweaver–Burke plots.

Protein concentrations in both binding and uptake experiments were determined using the method of Lowry et al. (1951).

Data analysis

The results are expressed as the mean ± SEM. Analysis of equilibrium binding data was performed using the BDATA computer program (EMF Software, Inc., Knoxville, TN, U.S.A.). Linear regression lines were fitted using a weighted least-squares method and Na^+-dependence curves were fitted to a rectangular hyperbola by a nonlinear least-square computer program. One-way analysis of variance (ANOVA) or Student's *t* test was used to calculate the statistical difference between the means whenever appropriate.

RESULTS

Sodium dependence of [^{3}H]paroxetine binding

In membrane preparations from rat diencephalon, [^{3}H]paroxetine binds to a single population of sites. Scatchard analysis of saturation binding yielded a linear plot with a mean K_D value of 0.28 ± 0.04 n*M* and B_{max} of 500 ± 36 fmol/mg of protein (n = 4) at the optimum (60 m*M*) NaCl concentration (Fig. 1A). Analysis of equilibrium binding of [^{3}H]paroxetine at various sodium concentrations in the incubation medium reported in Fig. 1A shows that [^{3}H]paroxetine binding saturates at each external Na^+ concentration and that virtually the same number of sites is available at each Na^+ concentration tested. Decrease in sodium concentration failed to alter the calculated B_{max} values (Fig. 2A). In contrast, the affinity ($1/K_D$) of [^{3}H]-paroxetine for its binding sites was significantly affected by altered external Na^+ concentration (ANOVA, $F = 3.995$, $p < 0.022$). By decreasing the Na^+ concentration from 60 to 15 m*M*, the K_D of [^{3}H]-paroxetine increased from 0.28 to 0.51 ± 0.02 n*M* (n = 4). The relationship of external Na^+ concentration to the change in affinity of [^{3}H]paroxetine binding ($1/K_D$) illustrated in Fig. 2A can be described as a simple rectangular hyperbola ($r = 0.924$, $p < 0.05$) to be expected when one Na^+ is required for the binding of one [^{3}H]paroxetine molecule.

The sodium requirement was also analyzed by studying the binding of [^{3}H]paroxetine at a single concentration (0.25 or 0.4 n*M*, respectively) to membrane preparations and coronal sections from rat diencephalon at varying sodium concentrations. The results reported in Fig. 3 show that the binding increased significantly with increasing sodium concentrations to reach a plateau at 60 m*M*. Their relationship in both cases was again best described by a rectangular hyperbola. The EC_{50} of sodium for the stimulation of [^{3}H]paroxetine binding in this experimental condition was ~8 and ~25 m*M* in membranes and sections, respectively. The double reciprocal plot of the sodium-dependence curve of [^{3}H]-paroxetine in membrane preparation (Fig. 3, inset) yielded a linear regression line ($r = 0.996$, $p < 0.005$).

Sodium requirement for [^{3}H]5-HT uptake

Data presented in Fig. 1B demonstrate the effect of varying external sodium concentration on the kinetics of [^{3}H]5-HT uptake by synaptosomes of brain diencephalon. As sodium concentration in the medium increases, the uptake saturates at lower serotonin concentrations and shows an increased initial transport rate (V_{max}). Both the K_m and V_{max} of [^{3}H]5-HT uptake showed significant changes due to varying sodium concentration (ANOVA, $F = 7.299$, $p < 0.012$, and $F = 6.357$, $p < 0.017$, respectively). The relationship between the external sodium concentration and the affinity of [^{3}H]5-HT for its transporter ($1/K_m$) as well as the rate of uptake (V_{max}) is reported in Fig. 2B. Both the affinity and transport rate increase by about two- to threefold with elevating the sodium concentration from 15 to 120 m*M*. Points on both curves fit a rectangular hyperbola ($r = 0.946$, $p < 0.02$ for $1/K_m$; $r = 0.957$, $p < 0.02$ for V_{max}) and suggest that one sodium ion is required for both binding and subsequent translocation of serotonin. A similar hyperbolic relationship was found between external sodium concentration and uptake of 50 n*M* [^{3}H]5-HT (data not shown) with an EC_{50} for sodium of ~20 m*M*. Double reciprocal plot of the sodium-dependence curve of serotonin uptake in this experimental condition yielded a linear regression line ($r = 0.998$, $p < 0.002$).

DISCUSSION

The kinetic parameters of [^{3}H]paroxetine binding in rat diencephalon obtained in this study were close to those reported for rat brain (Habert et al., 1985; Laruelle et al., 1988; Edwards et al., 1991) or human brain (Laruelle et al., 1988), although other investigators have reported lower K_D values for human as well as rat brain (Marcusson et al., 1988; Lawrence et al., 1990). The higher K_D values in our experiment may have been due to a relatively large fraction of the radiolabel bound. Additional factors could be the differences in tissue preparation and assay conditions

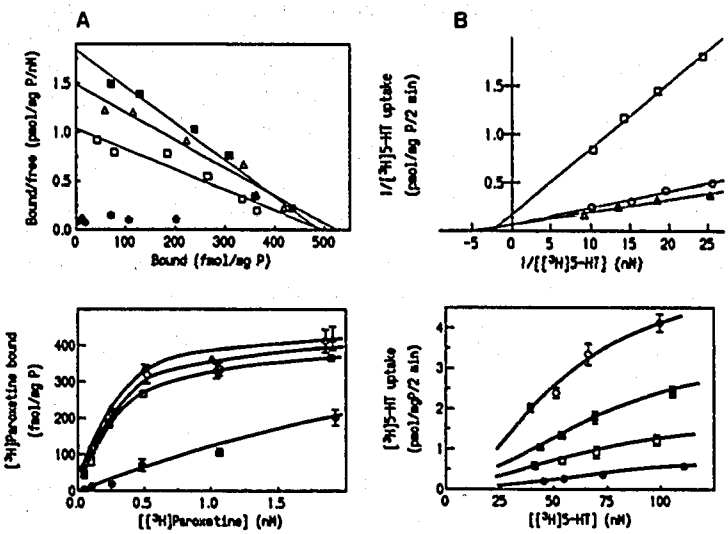

FIG. 1. A (Bottom): Saturation curves for specific binding of [^{3}H]paroxetine in membranes from rat diencephalon at increasing concentrations of sodium: 0 (●), 15 (□), 30 (Δ), and 120 (○) m*M*. Binding was determined over a concentration range of [^{3}H]-paroxetine of 0.05–2.0 n*M*. Each point represents the mean ± SEM of four separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol. **(Top):** Scatchard analysis of [^{3}H]paroxetine binding at 0 (●), 15 (□), 30 (Δ), and 60 (■) m*M* NaCl. The B_{max} values were 513 ± 17.5 (15 m*M* NaCl), 537 ± 35.3 (30 m*M* NaCl), and 500 ± 36.1 fmol/mg of protein (60 m*M* NaCl). The K_D values were 0.51 ± 0.02 (15 m*M* NaCl), 0.38 ± 0.05 (30 m*M* NaCl), and 0.28 ± 0.04 n*M* (60 m*M* NaCl). **B (Bottom):** Saturation curves for [^{3}H]5-HT uptake in synaptosomes from rat diencephalon at increasing concentrations of sodium: 0 (●), 15 (□), 30 (■), and 60 (○) m*M*. Each point represents the mean ± SEM of three separate experiments performed in triplicate. Where error bars are not shown, the SEM was smaller than the size of the symbol. **(Top):** Same data expressed in Lineweaver–Burke plots. Data shown are from experiments at 15 (□), 60 (○), and 120 (Δ) m*M* NaCl. The corresponding V_{max} values were 5.59 ± 0.972, 16.5 ± 2.64, and 16.0 ± 1.32 pmol/mg of protein/2 min. The corresponding K_m values were 0.43 ± 0.03, 0.28 ± 0.04, and 0.21 ± 0.01 μ*M*.

(amount of protein per tube, incubation volume, volume of nonspecific binding).

The sodium dependence of paroxetine binding had not been investigated before this study, although that of imipramine, a nonselective 5-HT uptake blocker, had been studied in both brain and platelets in terms of the nature of sodium requirement for binding. Talvenheimo et al. (1983) found no change in the B_{max} of [^{3}H]imipramine binding in platelets over increasing concentrations of sodium, although K_D increased significantly as sodium was reduced. In brain cerebrocortical membranes, Briley and Langer (1981) also found no change in the B_{max} of [^{3}H]imipramine binding and a high K_D value in the absence of NaCl. Both 1:1 and 1:2 ratios of imipramine molecules to sodium ions have been suggested as a requirement for [^{3}H]-imipramine binding (Talvenheimo et al., 1983; Wood, 1987). Kinetic evidence based on the linear double reciprocal plot of [^{3}H]imipramine bound and NaCl concentration led Wood (1987) to suggest the 1:1 ratio, whereas Talvenheimo et al. (1983) found a

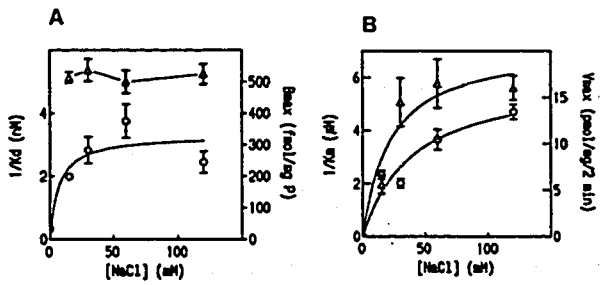

FIG. 2. A: Relationship of external sodium concentration and the affinity ($1/K_D$; ○) or density (B_{max}; Δ) of [^{3}H]paroxetine binding in membranes from rat diencephalon. Data are transformed from saturation binding experiments presented in Fig. 1A and yielded a good fit for rectangular hyperbola for $1/K_D$ ($r = 0.924$, $p < 0.05$). **B:** Relationship of external sodium concentration and the affinity ($1/K_m$; ○) or rate (V_{max}; Δ) of [^{3}H]5-HT uptake by synaptosomes from rat diencephalon. Data are transformed from kinetic experiments presented in Fig. 1B and best fit a simple rectangular hyperbola ($r = 0.967$, $p < 0.01$ for $1/K_m$, and $r = 0.957$, $p < 0.02$ for V_{max}).

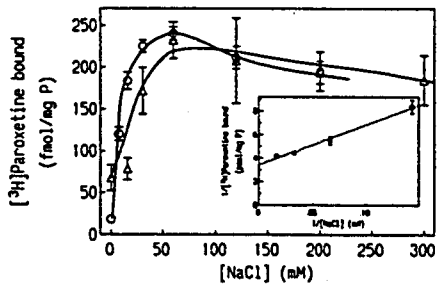

FIG. 3. Effect of increasing concentrations of sodium on specific binding of [^{3}H]paroxetine in membrane preparation (○) and coronal sections (Δ) from rat diencephalon. Specific binding was defined as the difference between binding in the absence (total) or presence (nonspecific) of 10 μ*M* fluoxetine. Binding in homogenates was measured at 0.25 n*M* and in sections at 0.4 n*M* [^{3}H]-paroxetine. Each point represents the mean ± SEM of four separate experiments performed in triplicate (homogenates) or duplicate (sections). Where error bars are not shown, the SEM was smaller than the size of the symbol. **Inset:** Double reciprocal plot of 0.25 n*M* [^{3}H]paroxetine binding in membranes from rat diencephalon and external sodium concentration ($r = 0.996$, $p < 0.005$, for the linear regression line). Data transformed from the binding curve (points until saturation) are shown in this figure.

rectangular hyperbolic relationship between [^{3}H]-imipramine affinity ($1/K_D$) and [Na^+][2] suggesting that two sodium ions are required for binding of one imipramine molecule. However, the findings of sodium dependence of imipramine binding cannot simply be extended to the binding of paroxetine because paroxetine, in contrast to imipramine, appears to label a single class of high-affinity sites (Marcusson and Eriksson, 1988).

In the present study, the density of [^{3}H]paroxetine sites (B_{max}) was independent of sodium concentration. This is similar to findings with [^{3}H]imipramine and some other ligands that label amine transporters, e.g., [^{3}H]mazindol (Zimanyi et al., 1989). In contrast, the affinity of [^{3}H]paroxetine for its binding sites was clearly sodium dependent. The kinetic analyses of the relationship between sodium concentration and the affinity of binding ($1/K_D$) or the amount of binding at single concentrations of ligand strongly suggest that one sodium ion is required for binding of the paroxetine molecule to the transporter. This is similar to the sodium requirement for high-affinity binding of [^{3}H]-imipramine in brain tissue (Wood, 1987), and for [^{3}H]5-HT binding and translocation in brain and platelets (Talvenheimo et al., 1983; Wood, 1987; present data). The results thus further support the single-site model of the neuronal 5-HT uptake and imipramine-binding site originally suggested by Marcusson et al. (1986). The sodium dependence of V_{max} of [^{3}H]5-HT uptake, in contrast to B_{max} of [^{3}H]-paroxetine binding, probably reflects the sodium requirement of the translocation step that follows the binding of 5-HT to its recognition site.

The V_{max} of [^{3}H]5-HT uptake in platelets was found to be clearly sodium dependent at ≤200 m*M* NaCl (Talvenheimo et al., 1983); we found a similar sodium dependence of V_{max} in rat diencephalon. The sodium dependence of the K_m of 5-HT uptake has been controversial in past reports. K_m was demonstrated to be significantly sodium dependent in human and rat platelets (Sneddon, 1969; Talvenheimo et al., 1983) and in guinea-pig striatal tissue (Kilpatrick et al., 1986). In agreement with these findings, we found the K_m of [^{3}H]5-HT uptake to be significantly sodium dependent in the rat diencephalon. In contrast, Wood (1987) reported no sodium dependence of the K_m of [^{3}H]5-HT uptake in rat brain cortex. However, his kinetic analysis of the effect of sodium on the uptake of 100 n*M* [^{3}H]5-HT yielded a linear regression line indicating that the transport of one 5-HT molecule was associated with the interaction of one sodium ion. We have obtained a similar linear regression using 50 n*M* [^{3}H]5-HT concentration. The hyperbolic relationship between external [Na^+] and both uptake affinity ($1/K_m$) and rate (V_{max}) suggests that one sodium ion is required for both binding and subsequent translocation of serotonin. Recently, O'Reilly et al. (1988) suggested that in membrane vesicles from mouse cerebral cortex, two sodium ions are required for serotonin uptake. This finding is at variance with the sodium requirement for 5-HT uptake in rat diencephalon (present study) and human platelets (Talvenheimo et al., 1983). The reason for this discrepancy may be in the preparations, tissues, or species examined. Direct estimation of the number of sodium ions actually cotransported together with serotonin may have to be used to resolve this discrepancy. The recent cloning and expression of a functional serotonin transporter from rat brain (Blakely et al., 1991; Hoffman et al., 1991) may in the near future facilitate further studies to elucidate the nature of 5-HT transport and of the molecular pharmacology of this transporter.

In conclusion, [^{3}H]paroxetine binding and [^{3}H]5-HT uptake in brain diencephalon have similar requirements for external sodium. The binding of [^{3}H]-paroxetine as well as the binding and subsequent translocation of [^{3}H]5-HT both require 1:1 ratios to sodium ions. This finding concurs with a single-site model of the sodium-dependent serotonin neuronal transporter with common or overlapping domains on the substrate recognition site that would bind serotonin and the tricyclic and nontricyclic 5-HT uptake inhibitors.

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ABSTRACT

MECHANISMS OF ACTION OF ANTIDEPRESSANT DRUGS: PRESYNAPTIC AND POSTRECEPTOR MECHANISMS

The etiology of clinical depression is unknown, but thought to be related to an impairment in brain transmission of monoamines. Depression is treated with antidepressant drugs which, regardless of classification, ultimately result in increased efficacy of aminergic transmission. Tricyclic antidepressants are known to inhibit the presynaptic uptake of amines. [^{3}H]Imipramine, the prototype tricyclic antidepressant and a potential biological marker in depression, binds to both high- and a low-affinity sites in the brain. The high-affinity binding of [^{3}H]imipramine to its binding site on the serotonin (5-HT) neuronal transporter has been shown to be a sodium-dependent process. However, that of [^{3}H]paroxetine, a novel and selective 5-HT uptake inhibitor, has not yet been investigated. Because of a discrepancy between the onset of uptake-blocking effect and alleviation of depressive symptoms, the blockage of uptake is probably not the only pharmacological action of antidepressants underlying their clinical effect. Recent studies have reported decreases in ß-adrenoceptor and 5-HT$_2$ receptor numbers following long-term treatment with antidepressants, and suggested that an adaptive change in amine receptors may be more relevent for the clinical effect. However, not all 5-HT uptake inhibitors elicit this downregulation. Recent research has thus centred on elucidating changes in the signal transduction apparatus of aminergic neurons. The 5-HT$_2$ receptor in the brain is coupled to the phosphoinositide turnover cascade and protein kinase C (PKC) activity. The subcellular distribution of PKC following chronic antidepressant treatment has not yet been investigated.

This study was undertaken 1) To compare the sodium dependence of [^{3}H]paroxetine

binding and [^{3}H]5-HT uptake in rat diencephalon in order to confirm whether paroxetine binds to the 5-HT recognition site on the transporter, and 2) to investigate the effects of both acute and chronic antidepressant treatment on PKC location and activity (both basal and 5-HT_2 receptor agonist (DOI)-challenged) in rat cortex and hippocampus.

The sodium dependence of [^{3}H]paroxetine binding in both homogenates and sections and of [^{3}H]5-HT was assessed by determining the kinetic parameters of these two processes in the presence of increasing concentrations of NaCl. Binding of [^{3}H]paroxetine in both membranes and sections displayed clear sodium dependence until a plateau occurring at 60 mM NaCl. The affinity (1/Kd) of [^{3}H]paroxetine binding was a simple hyperbolic function of sodium concentration. In contrast, the density of [^{3}H]paroxetine sites was not affected by external sodium concentration. Uptake of [^{3}H]5-HT showed a pattern of sodium dependence similar to that for [^{3}H]paroxetine binding affinity. Both the affinity (1/Km) and the rate (Vmax) of [^{3}H]5-HT uptake were dependent on external sodium with sodium-dependence curves fitting a rectangular hyperbola. The kinetic analysis of results indicates that one sodium ion is required for the binding of [^{3}H]paroxetine as well as for the binding and translocation of each [^{3}H]5-HT molecule. The results concur with a single-site model of the sodium-dependent serotonin transporter with common or overlapping domains for 5-HT and 5-HT uptake inhibitors. Chronic treatment with desipramine caused no change in the parameters (Vmax, Kd) of [^{3}H]5-HT uptake. Chronic treatment with fluoxetine resulted in a decrease in rate (Vmax, by 42 %) and in affinity (1/Km, 77 % increase in Km) of [^{3}H]5-HT uptake.

PKC location in both subcellular fractions of brain homogenates as well as in sections was determined by measuring the specific binding of [^{3}H]PDBu, a phorbol ester which binds

and stimulates PKC. [^{3}H]PDBu binding in sections revealed a heterogenous distribution of PKC in the brain with highest binding in cortex and hippocampus. Acute desipramine treatment (10 mg/kg one hour before death) resulted in a decrease in [^{3}H]PDBu binding to brain sections, significantly lower than in controls in the inner 3 layers of parietal cortex (13 % decrease) and striatum (9 % decrease). [^{3}H]PDBu binding in homogenates from control animals amounted to 65.5 ± 3.73 and 12.1 ± 1.01 pmol/mg protein in soluble and particulate fractions of cortex, respectively, and to 55.3 ± 9.51 and 9.28 ± 1.21 pmol/mg protein in soluble and particulate fractions of hippocampus, respectively. Significant decreases in [^{3}H]PDBu binding in both subcellular fractions of cortex (37 % in soluble; 27 % in particulate) were observed following acute DOI treatment (10 mg/kg one hour before death). This was the first demonstration of PKC changes following *in vivo* administration of DOI. *In vitro*, DOI was reported to produce translocation of PKC from cytosol to membrane. Chronic desipramine treatment (10 mg/kg/day for 21 days) produced no changes in [^{3}H]PDBu binding in subcellular fractions of brain tissue.

PKC activity was measured by the incorporation of [^{32}P]ATP into a PKC-specific commercially-available target peptide. PKC activity in control animals amounted to 3.49 ± 0.12 and 1.36 ± 0.08 nmol/min/mg protein in soluble and particulate fractions of cortex, respectively, and to 4.35 ± 0.53 and 1.48 ± 0.15 nmol/min/mg protein in soluble and particulate fractions of hippocampus, respectively. Acute DOI treatment resulted in decreases in PKC activity in both subcellular fractions of cortex (23 % in soluble; 37 % in particulate) and in particulate fraction of hippocampus (44 %) (similar to changes in PKC location following DOI administration). Significant decreases in both cortical (33 %) and

hippocampal (43 %) particulate fraction PKC activity were observed after acute desipramine treatment. Acute fluoxetine treatment (5 mg/kg one hour before death) induced similar changes in PKC activity as those observed after acute DOI treatment (23 % and 48 % decreases in soluble and particulate fractions of cortex; 36 % decrease in particulate fraction of hippocampus); the changes may be due to increased synaptic serotonin content with resultant increased activity of 5-HT_2 receptors, hence eliciting the same effect as acute DOI. Chronic desipramine treatment produced decreases in basal PKC activity in soluble fraction of cortex (38 %), and in DOI-challenged PKC activity in soluble fraction of hippocampus (26 %), implying a functional sensitization to 5-HT agonist. Chronic fluoxetine treatment (5 mg/kg/day for 21 days) led to significant decreases in basal PKC activity, in both subcellular fractions of both tissues (47 % and 33 % in soluble and particulate fractions of cortex, respectively; 31 % in both fractions of hippocampus), and significant increases in DOI-challenged PKC activity in both subcellular fractions of both tissues (178 % and 145 % in soluble and particulate fractions of cortex, respectively; 104 % and 133 % in soluble and particulate fractions of hippocampus, respectively); these increased responses could be due to a presynaptic action of fluoxetine on PKC.

These results indicate that, antidepressant drugs induce differing but significant effects on PKC activity in the subcellular fractions of neurons both after acute and chronic treatment. These changes in PKC activity may alter transduction of cellular signals evoked by the binding of 5-HT to receptors.