National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

Fos-Like Immunoreactivity in Forebrain Regions Following Self-Stimulation of the Lateral Hypothalamus and Ventral Tegmental Area.

Cecilia Flores

A Thesis

in

The Department

of

Psychology

Presented in Partial Fulfillment of the
Requirements for the degree of Master of Arts at
Concordia University
Montréal, Québec, Canada

November, 1995

ISBN 0-612-10848-1

Canada

ABSTRACT

Fos-like immunoreactivity in forebrain regions following self-stimulation of the lateral hypothalamus and ventral tegmental area.

Cecilia Flores

Identification of the directly-activated neurons underlying self-stimulation of the medial forebrain bundle (MFB) is a major goal of contemporary research on the neural basis of reward. Psychophysical, electrophysiological, and anatomical evidence suggests that the somata of some of the directly-activated cells subserving the rewarding effect are located in basal forebrain nuclei and that some of their fibers link the lateral hypothalamus (LH) and ventral tegmental area (VTA). In the present study, Fos-like immunoreactivity (FLIR) in forebrain regions was assessed following self-stimulation of the VTA and LH in male rats. The 0.5 s stimulation trains were composed of 0.1 msec, 1000 μA pulses, and the stimulation frequency for each subject was chosen so as to fall on the shoulder of the rate-frequency curve. The rats were sacrificed 15 min after a final 1 h self-stimulation session. Following perfusion and debraining, vibrotome sections were cut and processed for FLIR. Two subjects served as controls. One of them was trained and

tested in the same manner as the experimental subjects, except that the stimulator was disconnected during the last 1 hour test. The other did not undergo any manipulation prior to perfusion. Among the regions where FLIR was greater in the stimulated hemisphere following either LH or VTA stimulation were the anterior LH (LHA), the substantia innominata (SI), and the bed nucleus of the stria terminalis. Lesions of at least two of these regions, the LHA and the SI, have been shown to decrease the rewarding effectiveness of both LH and VTA stimulation.

Acknowledgments

I am grateful to my supervisor Peter Shizgal for accommodating me in his lab with all the necessary means to accomplish my work. I have benefited enormously from his ideas, guidance, and valuable criticism. It was a privilege to work with such a good scientist.

I am also deeply indebted to Jane Stewart. A great part of my achievement is due to her help, confidence, and support. I have learned innumerable things from her and I consider myself fortunate to know her.

Barbara Woodside was of great assistance with the statistical analysis. I am thankful for her kindness, friendliness, and generosity.

I want to thank Jim Pfaus for his comments, his accessibility, and his good cheer.

I wish to express my gratitude to Roy Wise for his advice and help with the bibliography, and to Shimon Amir for lending me his expertise with the image analysis software and much of his equipment.

I also want to acknowledge the members of the lab: Kent Conover, Luigi Riscaldino, and Peter Marinelli for all their

help, particularly with the rats (i.e. frequent trips to the 14th floor), and for providing me with a pleasant and supporting environment.

Finally, my heartfelt gratitude to Andreas for so many things: our frequent discussions, his help in technical matters, his patience and support, but specially for his encouragement through good times and bad.

Dedication

This thesis is dedicated to the memory of my father Guillermo, to my dear mother Bebé, my sister Carolina, and my brothers Guillermo and Federico. I am forever grateful for their love and support.

Table of contents

List of Figures

Organisms are constantly faced with situations requiring actions, often mutually exclusive, towards various goals present in the environment. In order to survive, the value of different biological relevant goals, say food or water, has to be adjusted to reflect current internal and external demands. Therefore, the rewarding value of a biological object should vary depending on its cost and availability and on the organism's internal state. To understand the adaptive selection of goals, it is essential to identify the neural substrate(s) underlying reward.

The discovery of the phenomenon of self-stimulation by Olds and Milner in 1954 provided for the first time an animal model for studying the physiological basis of goal directed behavior. They demonstrated that electrical stimulation of certain parts of the limbic system has a powerful reinforcing effect. Rats could be shaped to produce a response, such as lever-pressing, in order to obtain electrical stimulation. Numerous studies have established the occurrence of this phenomenon in different species, ranging from snails to humans. Others showed that animals are willing to self-administer the stimulation even at the expense of satisfying their primary physiological needs (Routtenberg & Lindy, 1965). These findings have led investigators to hypothesize that the brain circuitry underlying intracranial self-

stimulation is also responsible for homeostatic appetitive behaviors that guide animals towards naturally occurring goals such as food (Hoebel, 1969). In fact, it has been suggested that a single set of fibers accounts for both feeding and brain stimulation reward (BSR) (Gratton & Wise, 1988a, 1988b). More recently, Conover and Shizgal have assessed the relationship between BSR and naturally occurring reinforcers showing that the rewarding effects of brain stimulation and of gustatory stimuli are evaluated in a common system of units and combined agonistically (Conover & Shizgal 1994a; 1994b; Conover, Woodside & Shizgal 1994).

The idea of a common neural substrate for self-administration of electrical stimulation and dependence inducing substances has also been put forward (Wise, 1980). There is evidence of an overlap in the neural system involved in the rewarding effects produced by some classes of abused drugs and rewarding brain stimulation of at least some brain regions (Wise, 1991; Wise, Bauco, Carlezon & Trojniar, 1992). Firstly, systemic administration of psychomotor stimulants such as amphetamine (Gallistel & Karras, 1984; Wise & Munn, 1993) as well as opiates (Wise & Bozarth, 1982; Rompré & Wise, 1989) enhances BSR. Secondly, electrical and chemical recordings demonstrated that self-administration of both electrical stimulation and substances of abuse activates

mesolimbic dopamine neurons (Blaha & Phillips, 1990; Nakahara, Fuchikami, Ozaki, Iwasaki & Nagatsu, 1992). Thirdly, several studies have shown that the administration of the neuroleptic pimozide hampers self-stimulation and blocks drug self-administration (Fouriezos & Wise, 1976; Gallistel & Karras, 1984). Lastly, intracranial injections of dopamine agonists and antagonists generally show that the most sensitive sites for these reward-related effects are at the cell bodies and terminals or the mesolimbic dopamine system (Colle & Wise, 1988; Bozarth and Wise, 1981).

Taken together, the above-mentioned studies show that the mesolimbic dopaminergic system could be a possible mediator of the rewarding effects of BSR and drug self-administration. However, a compelling amount of data has indicated that at least a large part of the directly activated substrate subserving the rewarding effects of electrical brain stimulation is not dopaminergic (Shizgal & Murray, 1989). It is well accepted that dopamine plays a role in BSR, albeit serving primarily as a modulatory neurotransmitter (Gallistel, Shizgal & Yeomans, 1981). The neurotransmitter(s) directly involved in the rewarding effects of BSR still remains to be identified. The experiments described below are aimed at localizing neurons activated during BSR so as to bring us a step closer towards

identifying the neurochemical nature of the reward-relevant circuitry. Knowing the type of neurochemicals involved in the rewarding processes will provide the basis for pharmacological manipulations that will likely contribute to our understanding of motivated behavior and might conceivably contribute to the development of therapies for drug abuse.

A reasonable place to begin the search of the neural substrate subserving BSR, is at the electrode tip. The neurons that process or *translate* the electrical stimulation into a meaningful signal must pass close to the electrode tip. Localization of the directly stimulated somata that are responsible for self-stimulation ('first-stage' neurons) would constitute the first step towards defining the reward-relevant pathway. Insofar as the identity of the first-stage cells is recognized, tracing the circuitry in which they are embedded will become a realistic task. Many of the brain loci that support self-stimulation, as identified by mapping studies, send fibers that course through the medial forebrain bundle (MFB). In fact, self-stimulation of some of the sites localized along the trajectory of this bundle, for example, the lateral hypothalamus (LH) and the ventral tegmental area (VTA), produce vigorous and reliable performance. Not surprisingly, much research on BSR has been concentrated on identifying the first-stage neurons within the MFB. Yet,

already in the fifth decade since the discovery of BSR, the identity of the first-stage cells is still unknown.

The MFB is a complex system that comprises at least 50 ascending and descending populations of fibers, of different origins and destinations, most clearly defined as they traverse the lateral preoptic and lateral hypothalamic areas (Nieuwenhuys, Geeraedts & Veening, 1982). Given its heterogeneity, it is likely that an electrode placed in the bundle will activate more than one set of cells, including the first stage-neurons as well as cells that do not contribute to the rewarding effects of electrical stimulation.

One of the most important approaches developed to differentiate first-stage cells from all neurons directly activated by the electrode uses psychophysical methods. Physiological and anatomical properties of the substrate for self-stimulation have been estimated by systematically varying the parameters of the stimulation and measuring the behavioral consequences. These studies have provided information about the general characteristics of the first-stage neurons and about their possible location and neurochemical features.

Physiological and anatomical characterization of the first-stage neurons

By means of behavioral trade-off experiments, the excitability properties, the fiber diameter, the conduction velocity, and the direction of conduction of the first-stage neurons have been described. In these experiments the value of one parameter of stimulation required for the rat to reach a criterion level of performance is assessed when another parameter is manipulated. For instance, if the current is varied, the number of pulses have to be adjusted so as to produce a constant rate of lever-pressing. A function that relates stimulation current and frequency characterizes important properties of the anatomical substrate under study. In fact, the current-frequency function has revealed that to a first approximation these two parameters are inversely related. The stimulation frequency pertains to the number of times a neuron fires whereas the stimulation current pertains to the number of neurons fired. The current-frequency function shows that the product of current and pulse frequency is approximately a constant. This finding indicates that regardless of the spatiotemporal distribution, the number of action potentials generated by the population of reward-relevant neurons determines the reward magnitude of the electrical stimulation when the train duration is fixed.

The spatial and temporal integration of the output of the first-stage neurons has been characterized as a 'counter model' (Gallistel et al., 1981).

The refractory periods of the first-stage neurons have been inferred from the results of double pulse experiments, in which the number of pulse pairs required to produce a criterion rate of lever-pressing is traded off against the intra-pair interval. When two pulses, the conditioning (C) and the testing (T) pulse, are delivered through the same electrode, the interval between them will determine the effectiveness of the second pulse to generate an action potential. In other words, if the time interval is too short, the axon of the stimulated cell will not have enough time to recover from refractoriness and the second pulse will not succeed in firing the cell again. Thus, in order for the rat to maintain a certain criterion of lever-pressing a higher number of single pulses will be required. The effects of paired-pulse effects are scaled as a function of the required number of single pulses necessary to maintain the criterial behavior. This method, developed by Yeomans (1979), provides an estimate of recovery from refractoriness in the directly activated neurons mediating the rewarding effects of MFB self-stimulation.

A powerful psychophysical method that has furnished us with valuable information about the physiological and anatomical characteristics of first-stage neurons is the collision technique (Shizgal & Murray, 1994). From collision tests it is feasible to make inferences about conduction velocity of a particular set of fibers, and about anatomical linkage between two stimulated sites. Collision follows the logic of double pulse studies, taking into account the fact that an action potential propagates both orhtodromically and antidromically along the trajectory of an axon. Rather than applying the pair of pulses through the same electrode, the C and T pulses are delivered through two different electrodes at two sites that support BSR. For simplicity's sake, suppose that the two electrodes activate one and the same fiber. In that case, if the two pulses are delivered almost simultaneously, the orthodromic action potential fired by the electrode closer to the soma will 'collide' with the antidromic action potential generated by the electrode further from the soma and they will cancel each other. When collision occurs, the action potential triggered by the electrode closer to the soma will not reach the terminal of the axon, and will not have any behavioral effect. Simply put, at short C-T intervals, only one pulse will be effective. In situation where a fiber bundle instead of a single fiber is stimulated, at short C-T intervals a higher

number of single pulses will be needed in order to meet the chosen behavioral criterion. In the case of self-stimulation, collision between action potentials delivered to two different sites happens only when reward relevant fibers link the two sites.

Shizgal, Bielajew, Corbett, Skelton and Yeomans, (1980) reported collision between the LH and VTA, thus demonstrating that some of the fibers of the first-stage are continuous between these two structures. Furthermore, they derived the conduction velocity of the first-stage fibers using the obtained estimates of collision interval and refractory period as well as the interelectrode distance (Bielajew & Shizgal, 1982) The conduction velocity estimates for the LH-VTA placements were shown to be compatible with the properties of small myelinated fibers ranging from 0.5-2.5 μm in diameter. Fibers of the same caliber are known to be part of the MFB (Nieuwenhuys et al., 1982). More recently, collision effects have been obtained from self-stimulation sites in the anterior LH (LHA) and VTA (Murray & Shizgal, in press,a).

Collision studies provide a means for identifying anatomical linkage between two self-stimulation sites but they do not tell us about the localization of the somata that

give rise to the activated fibers. Are the reward relevant fibers ascending or descending? Knowing the direction of conduction of these fibers is essential to localize where in the brain these neurons originate. Bielajew and Shizgal (1986) employing a modification of the collision technique whereby one of the electrodes served to produce an anodal hyperpolarization block, showed that for at least some of the directly activated fibers subserving MFB reward the behavioral relevant direction of conduction is rostrocaudal. The idea that at least part of the first-stage neurons arise in the forebrain and give rise to fibers that descend through the MFB towards the tegmentum is referred to as the 'descending path hypothesis.'

Taken together, the data derived from the psychophysical studies provided researchers with a portrait of the substrate for BSR. In sum, the directly stimulated substrate consists, at least in part, of thin myelinated fibers with short refractory periods and relatively fast conduction velocities which in most likelihood originate in the forebrain and descend through the MFB to at least the VTA. If one identifies by means of electrophysiological recordings structures containing neurons that match the psychophysically derived properties of the first-stage neurons, then, lesion studies could be conducted to determine whether the nuclei

identified play a role in BSR. At this point it should be mentioned that the psychophysical data seem to rule out the assumption that dopaminergic neurons are the first-stage cells. The conduction velocity estimates for the first-stage neurons are several times faster than those of the dopaminergic cells (Yim & Mogenson, 1980). Moreover, dopaminergic neurons ascend rather than descend through the MFB (Lindvall & Björklund, 1974).

Basal forebrain: locus of the first stage neurons?

Are there, indeed, cells in the forebrain that possess the anatomical and physiological characteristics expected from first-stage neurons? By means of acute single unit recordings performed in anesthetized rats, Rompré and Shizgal (1986), and Murray and Shizgal (in press,b) found that some cells arising in the forebrain can be antidromically activated by the stimulation of the LH and VTA. Among those cells, some exhibited refractory periods that overlapped with the ones described for the first-stage neurons and could thus be considered 'candidate' cells. From the different regions sampled, candidate neurons were found in the septum (SEPT) and in basal forebrain nuclei such as the medial preoptic area (MPOA), lateral preoptic area (LPOA), substantia innominata (SI), olfactory tubercle (OT), and bed nucleus of

the stria terminalis (BST). In the above-mentioned studies there was no way to assess whether the stimulation electrodes supported self-stimulation. To address this problem, Shizgal, Schindler, and Rompré (1989) collected psychophysical and electrophysiological data from the same subjects using same electrodes and stimulation fields in the behavioral and electrophysiological stages of the study. They found candidate neurons in the basal forebrain that were antidromically activated by rewarding stimulation of more caudal MFB sites.

Unfortunately, one cannot conclude that a candidate cell is part of the first-stage merely because it has the attributes required of a reward-relevant neuron. It is possible that rewarding stimulation of the MFB excites neurons that play no role in BSR, but happen to have electrophysiological characteristics similar to those required of the first-stage cells. A straightforward approach to find out if candidate cells play a role in BSR would be to damage structures that contain candidate neurons. If the rewarding impact of the stimulation is decreased after the lesion, it is likely that at least some of neurons within those structures are relevant to BSR.

Several lesions studies have been conducted since the discovery of BSR. However, in most of them the methods used to measure the lesion effects yield results that cannot be interpreted unambiguously. More specifically, the manner in which data were collected made it impossible to distinguish changes resulting from a degradation of the reward effectiveness of the stimulation from those due to lesion-induced motor deficits. In modern lesion studies the rate-frequency curve shift paradigm (Edmonds & Gallistel, 1974) has been implemented in an attempt to dissociate the two distinct effects. Lesion effects are quantified in terms of changes in the amount of stimulation required to sustain some criterion level of lever-pressing, for example, half-maximal responding. By keeping the current constant and varying the stimulation frequency, the performance of a rat will range from no responding to an asymptotic rate of lever-pressing. The rate of lever-pressing can then be plotted as a function of stimulation frequency so as to yield a sigmoid that is best described by a broken-line function (Gallistel & Freyd, 1987). In lesion experiments, rate-frequency curves are plotted before (baseline condition) and after the lesion and lateral displacements along the frequency axis are compared between the two conditions. In the case of an effective lesion the rate-frequency curve is displaced to the right along the frequency axis because a higher frequency will be

needed in order for the animal to sustain criterial responding. On the contrary, if the lesion simply induces motor deficits without affecting reward, a decrease in the asymptote of the rate-frequency curve will be observed. It is important to mention that this decrease may be accompanied by an increase in the frequency domain of the rising portion of the rate-frequency curve, referred to as the dynamic interval (Milliaressis, Rompré, Laviolette, Phillippe & Coulombe, 1986). An increase in the dynamic interval would tend to inflate our estimate of the lateral position of the curve. When dynamic interval and maximum rate analysis are carried out in conjunction with the rate-frequency curve shift, the above-mentioned paradigm represents a useful method of assessing lesion effects.

To find out the role that forebrain regions play in BSR Waraczynski (1988), used the rate-frequency curve shift paradigm to assess the effects of coronal knife cuts in a variety of septal and basal forebrain regions on the rewarding effects of LH self-stimulation. She observed that cuts in the medial septum failed to produce consistent, long lasting decreases in the effectiveness of the stimulation. However, in some of her subjects with cuts in the LPOA, moderate but long lasting decreases in the rewarding effects of LH self-stimulation were produced. Similarly, Janas and

Stellar (1987) found that self-stimulation of the LH was challenged by knife cuts in the caudal portion of the LPOA.

The logic underlying knife cut lesions is to transect neural pathways and separate rostral regions from caudal ones. Although Waraczynski's data contested the descending path hypothesis, the positive effects pertaining to cuts of the LPOA suggested that further studies be done to test the importance of this and more posterior brain sites in BSR. Electrolytic lesions of the anterior MFB were carried out by Murray and Shizgal (1991, in press,c). They observed rightward shifts in the rate-frequency curve after lesioning the border of the LHA and LPOA, and the SI. It is important to mention that in some subjects these lesions were effective in decreasing the rewarding effects of self-stimulation of both the LH and VTA. In contrast, electrolytic lesions to the amygdaloid complex (AMY) (Waraczynski, Ng Cheong Ton & Shizgal, 1990) and dorsomedial hypothalamus (DMH) (Waraczynski, Conover & Shizgal, 1992) failed to produce rightward shifts for either LH or VTA self-stimulation.

In spite of the ample use of electrolytic lesions as a means of damaging neural tissue, results stemming from their use cannot be interpreted unambiguously. The attenuation of the rewarding effects of self-stimulation by means of

electrolytic lesions does not necessarily imply that the destruction of cell bodies lying in the damaged area is the only factor responsible for the observed effects. The destruction of fibers of passage coursing through the lesioned area could also be causing the degradation of MFB reward. Excitotoxic lesions have the potential to discern the most likely of the above mentioned scenarios. By acting on specific receptors in the somatodendritic region of neurons, excitotoxins kill cell bodies while leaving the axons of passage intact. Stellar, Hall and Waraczynski (1991) examined the effects of ibotinate lesions on the intrinsic cells of the LH on BSR and found no or little changes in the rate-frequency curve. Arvanitogiannis, Waraczynski and Shizgal (in press) assessed the contribution of somata localized in the anterior MFB to rewarding LH and VTA stimulation using N-methyl-D-aspartate (NMDA) lesions. Damage encompassing the LPOA, LHA and SI resulted, in many cases, in long lasting, large increases in the frequency required to sustain half-maximal responding. The majority of these effects were restricted to self-stimulation of the LH, although modest decreases in reward efficacy were observed in two animals self-stimulating via VTA electrodes. Varying degrees of demyelination accompanied the effective lesions, except in one subject.

To summarize, most of the results from lesion studies are in agreement with the idea that at least some of the reward relevant neurons are localized in anterior MFB regions similar to those where electrophysiological recordings have demonstrated neurons with properties similar to those expected from neurons composing the first-stage. Yet, more evidence is necessary before one can conclude that those structures in fact contain the first-stage somata. An effective lesion does not necessarily mean that the structure damaged has cell bodies that give rise to directly-stimulated fibers. One can always argue for a transsynaptic effect whereby the damaged cells are neurons that modulate transmission in the efferent pathway of the first-stage. In this regard, it becomes crucial to employ techniques with the power to visualize the cells that are indeed directly activated by the electrode. Moreover, this complementary approach will allow us to attach morphological and eventually neurochemical characteristics to these cells.

Fos expression: visualization of first-stage neurons

Proto-oncogenes are the cellular counterparts of viral transforming genes. Their discovery led to a new way of thinking about the molecular mechanisms that link short lived events to long term sequelae after extracellular stimulation.

It is well established that two types of responses occur when neurons are activated by external stimuli. The first response is rapid and transient and does not require protein synthesis at the time. In contrast, the second response, triggered by the transient one, has a more persistent action and its occurrence depends upon gene transcription. Elevated concentrations of intracellular calcium and the manufacturing of membrane receptors are, respectively, examples of the two kinds of responses. Several experiments have demonstrated that proto-oncogenes are rapidly and transiently induced by a variety of external stimuli, including neurotransmitters and membrane depolarization (Roberston, 1992). As a result, these genes have been called immediate early genes (IEG). The ability of IEGs to be induced by a cascade of events that do not include protein synthesis, distinguishes this class of genes from 'late response genes' whose induction depends on protein synthesis. Furthermore, 'late response genes' are likely to be targets of the protein products of some IEGs.

A wide variety of IEGs has been identified. Among the best characterized of these genes are *c-fos* and *c-jun* and their protein products Fos and Jun. Expression of *c-fos* and subsequent synthesis of Fos in the central nervous system have been reported after many types of stimulation including: electrical (Dragunow & Robertson, 1987), photic (Kornhauser,

Nelson, Mayo & Takahashi, 1990), nociceptive (Gogas, Levine & Basbaum, 1993), sexual (Pfaus, Jakob, Kleopoulos, Gibbs & Pfaff, 1994), and chemical (Moreli, Carboni, Cozzolino, Tanda, Pinna & Di Chiara, 1992). Many experiments, both in vivo and in vitro, have been conducted in order to identify the regulation and function of *c-fos* and other IEGs. It is established that stimulus-related events such as depolarization induce transcription of IEGs through the activation of second messenger systems. In the particular case of *c-fos* transcription, calcium entry is the major second messenger involved, although in some cells, *c-fos* can be induced by neurotransmitters that use either calcium or cyclic-adenosine monophosphate (cAMP) as their intracellular messengers (Sheng & Greenberg, 1990). It has been demonstrated that the protein product of *c-fos* interacts with the protein product of *c-jun* forming a hererodimeric Fos-Jun transcription factor complex which binds with high affinity and specificity to a deoxyribonucleic acid (DNA) specific sequence known as the activator-protein 1 (AP-1) site. Binding of the Fos-Jun heterodimer to AP-1 DNA sites leads to the activation of nearby promoters. Unfortunately, the real picture is not as simple as the one just described. That is, there actually exist families of IEGs composed of several members. The *c-fos* and *c-jun* genes belong to the *fos* and *jun* families respectively. Recent findings have shown that the

protein products of members of these two IEGs families interact with each other to form heterodimers with different affinities for the AP-1 site as well as different effects on transcription of 'long term genes' (Sheng & Greenberg, 1990).

Although the precise function of IEGs and their protein products is still unclear, their discovery has provided neuroscientists with a powerful anatomical tool for linking structure and function. By means of molecular and immunohistochemical techniques it is now possible to detect neurons whose activity has changed as a result of external stimulation. Visualization of *c-fos* messenger ribonucleic acids (RNAs) can be achieved utilizing molecular methods such as in situ hibridization. However, more often, Fos immunohistochemical procedures are employed to detect activated cells. The use of Fos as an anatomical marker presents several advantages. Its nuclear localization offers cellular resolution lacked by other metabolic markers including 2-deoxyglucose (2-DG). This feature also allows labeling of this protein in conjunction with cytoplasmic markers of diverse neurotransmitters and/or axonal tracers. The expression of Fos is delayed by 30 to 45 minutes while gene transcription occurs within 5 to 10 minutes (Morgan and Curran, 1991). This delay represents an advantage of using Fos versus *c-fos* labeling particularly in studies in which

animal manipulation and handling have to take place after testing and just prior to sacrifice. Induction of *c-fos* caused by these factors will not be manifested as Fos expression and, therefore, any Fos labeling will solely reflect neuronal activation caused by the actual testing.

In spite of the broad range of stimuli that are able to induce *c-fos* , the expression of this gene has proven to be stimulus specific. For example, Jasmin, Gogas, Ahlgren, Levine and Basbaum (1994) reported that noxious and non-noxious stimuli induce different patterns of Fos expression in spinal cord neurons. This is in agreement with available anatomical and electrophysiological evidence. This experiment stresses the importance of comparing the desired manipulation with appropriate controls so as to prove stimulus specificity and rule out Fos induction due to confounding factors.

Taking into consideration the advantages of Fos immunohistochemistry, Arvanitogiannis, Flores, Pfaus and Shizgal (submitted for publication) used this technique to visualize neurons activated by rewarding stimulation of the lateral hypothalamus. High levels of Fos immunoreactivity were observed in the brains of rats that self-stimulated while insignificant amounts were detected in the brains of

unstimulated control subjects. Furthermore, substantial staining was observed ispsilateral to the stimulation electrode. In agreement with previous findings, basal forebrain regions, such as the LPOA, SI and LHA showed increased ipsilateral labeling of Fos protein in all of the experimental subjects.

Aim of the present study

Psychopsysical studies suggest that the fibers of at least some of the first-stage neurons are continuous between the LH and VTA levels of the MFB and that at least some of them arise from forebrain nuclei. With the framework of this knowledge, one would expect that rewarding stimulation of *both* LH and VTA activates antidromically common forebrain neurons that are at least part of the first-stage substrate. The present study takes advantage of available molecular tools to identify forebrain areas where cell bodies are activated by rewarding stimulation of both LH and VTA. The rationale adopted in this experiment was suggested by Glimcher and Gallistel (1989) in their attempts to use a retrograde tracer to visualize candidate first-stage neurons. Because of its numerous advantages, Fos immunohistochemistry was used to visualize activated cells. Stimulating electrodes were implanted at the LH or VTA in each animal.

Fos-like immunoreactivity (FLIR) was assessed on a predetermined set of forebrain and midbrain structures. Most of these structures were selected based on their previous association with BSR or on purely anatomical grounds. Since the stimulation was unilateral, a greater number of labeled cells was expected to be exhibited in the stimulated hemisphere. Therefore, comparisons between the number of cells showing FLIR ipsilateral and contralateral to the stimulating electrode were conducted.

Method

Subjects

14 male rats of the Long-Evans strain (Charles River Breeding Farms) weighing 400-500 at the time of the surgery served as subjects. Animals were housed individually in plastic cages with free access to food and water and were maintained on a reverse 12 hr: 12 hr light/dark cycle. All behavioral testing was conducted during the dark phase of the cycle.

Surgery

Animals were anesthetized with sodium pentobarbitol (Somnotol, 65 mg/kg i.p.) 15 minutes after administration of atropine sulphate to reduce mucous secretions, and were fixed in a stereotaxic apparatus. In six of the 14 subjects monopolar electrodes were bilaterally aimed at the LH. In seven of the remaining subjects monopolar electrodes were bilaterally directed at the VTA. The following flat-skull coordinates were used: LH: 2.8 mm posterior to Bregma, 1.7 mm lateral to the mid-sagittal sinus, and 7.8 mm below the dura mater; VTA: 4.8 mm posterior to Bregma, 1.0 mm lateral to the mid-sagittal sinus, and 7.2 mm below the dura mater.

The electrodes were made from 00 insect pins insulated with Formvar to within 0.5 mm of their rounded tips. Flexible stainless steel wires attached to male amphenol pins were soldered to the electrodes. Five jeweller's screws were imbedded in the skull to secure the electrode assembly. A stainless steel wire attached to a male amphenol pin was wrapped around one of the screws to serve as the current return. At the conclusion of the surgery, the male amphenol pins attached to both the electrodes and the ground wire were inserted into a 9-pin, externally threaded connector. The entire assembly was held together with dental acrylic cement. At the end of the surgery, rats were injected with Buprenorphine (Buprinex, 0.05 mg/kg) to reduce pain and were allowed to recover for several days before behavioral testing began.

Behavioral Testing

Training apparatus

Subjects were initially screened for self-stimulation in wooden boxes measuring 25 cm x 25 cm x 70 cm, with Plexiglas front panels and wire-mesh floors. A Lehigh Valley rodent lever extended from the center of the left wall, approximately 5 cm from the floor. The lever controlled a

microswitch that activated a stimulator. A flexible cable was attached to the 9-pin connector on the subject's head and plugged into a 7-channel, slip-ring commutator which provided the connection to the stimulator. A yellow key light was located 5 cm above the lever to indicate stimulus availability. Electrical stimulation consisted of fixed, 0.5 s trains of cathodal, rectangular pulses, lasting 0.1 msec each. It was produced by dual constant amplifiers (Mundl, 1980) and controlled by hand-set integrated circuit pulse generators. Stimulation parameters were monitored on an oscilloscope.

Training procedure

Before training the subjects to self-stimulate, noncontingent stimulation of 200 μA and approximately 40 Hz was delivered to either stimulating electrode. If signs of aversion were present (e.g. vocalization, withdrawal from the lever, defecation and jumping) screening was terminated, otherwise, animals were trained to self stimulate using conventional shaping methods. At the beginning of the training the current was set at 800-1000 μA and the frequency was varied so as to find the best combination of parameters that maintained reliable rates of responding and minimal motor effects. The current was lowered when stimulation-

induced seizure or stimulation-induced movements were observed. Both stimulating electrodes were tested, but only the one that produce forceful lever-pressing and no motor effects was chosen for the rest of the experiment. One subject, LH 7, exhibited stimulation-induced movements contralateral to the stimulation electrode but nonetheless was included in the experiment so as to increase the sample size.

Testing apparatus

Testing was performed in a computer-operated setup similar to the hand operated equipment used for training. Testing chambers consisted of Plexiglas boxes measuring 25 cm x 25 cm x 75 cm, with removable floors, and were illuminated by a single 40-W bulb. Two Lehigh Valley rodent levers were located on opposite walls, 5 cm from the floor and 5 cm from the nearest corner. A key light was located 3 cm above each lever, a yellow one on one side and a red one (not used in this study) on the opposite side. The test chambers were contained in 50 x 50 x 90 cm plywood boxes insulated with 2.5 cm of Styrofoam. Fans were placed at the back of the boxes to provide ventilation and to prevent external noises from disturbing rats during testing. A remote-controlled video camera allowed the experimenter to monitor the rats from an

adjoining room. Temporal stimulation parameters for each test cage were controlled by a microprocessor and were monitored on an oscilloscope

Testing procedure

Testing involved two phases. In the first phase, the threshold frequency, that is, the lowest stimulation frequency that supports near maximal lever-pressing was estimated as follows: At a constant current (determined for each animal during training), the number of lever presses per 30 s trial was recorded for a range of stimulation frequencies. The frequency was decreased after every trial in 0.05 $\log_{10}$ units steps and ranged from a value that produced maximum responding to one that yielded less than 10 responses per trial (criterion for quitting). Availability of the stimulation was signaled by the illumination of the yellow key. Each trial was preceded by 5 priming trains with parameters identical to those of the trains during that particular trial. Five curves relating response rate to number of pulses per train (rate-frequency curves) were collected and the threshold frequency was determined. The first curve was considered a warm-up and was not used to estimate the frequency threshold.

In the second phase of testing, rats were allowed to self-stimulate for an hour with the stimulation parameters fixed. The stimulation parameters used in this phase were the threshold frequency derived from the rate-frequency curves, and the current determined during training. Both parameters were fixed for each animal. Subjects completed at least one of these sessions and then were left, for a minimum of 48 hours, in their home cages. Twelve subjects were then tested again for one hour using the same fixed stimulation parameters. The remaining two subjects served as controls. One of them, the 'trained control', was treated in the same manner as the rest of the rats, except that in the last hour session the stimulator was disconnected. The other subject, the 'naive control', did not undergo surgery and remained in its home cage during the entire period of behavioral testing.

Fos immunohistochemistry

All animals received an overdose of sodium pentobarbitol (120 mg/kg) 15 min after the termination of the last one hour testing session. The naive control subject received the same anesthetic overdose in its home cage. Once subjects did not react to nociceptive stimuli, they were perfused transcardially with ice-cold phosphate buffered saline (300 ml) followed by ice-cold 4% paraformaldehyde (400 ml) in 0.1

M phosphate buffer, pH 7.2. Both solutions were perfused using a peristaltic pump. When perfusion was completed brains were removed and postfixated overnight in 4% paraformaldehyde (50 ml) at 4° C. Prior to slicing each brain, the side contralateral to the stimulating electrode was marked with a knife cut in the most ventral part and throughout the cortex. Serial, 50 µm coronal sections were cut from each brain using a vibrotome and deposited into the wells (30 sections per well) of a Plexiglas carrousel with a meshed base. The carrousel was seated on a Pyrex dish filled with ice-cold 0.9% Trizma-buffered saline (TBS), pH 7.6.

Fos immunohistochemistry was performed according to the ABC method (Hsu, Raine, and Fanger, 1981, Pfaus et al., 1994). A mouse monoclonal antibody raised against residues 4-17 of the 55 kD Fos protein N-terminal (NCI-BCB Repositorym Quality Biotech, Camden, NJ), diluted to 1:8000 with 0.3% Triton X-100 in TBS and 1% normal horse serum (Vector) was used as the primary antibody. Free floating sections from each well were transferred from the carrousel to a 5 ml immunoassay vial containing 1 ml of the primary antibody solution and were incubated for 48 hours at 4° C. To terminate incubation with the primary antibody, sections were transferred back to the carrousel and washed 3 times (5 min each) in cold TBS. Rinsed sections were then transferred to

the immunoassay vials containing 1 ml of a solution of rat adsorbed biotinylated anti-mouse antibody (Vector) diluted 1:33 with 0.3% Triton X-100 in TBS and 1% normal horse serum, and were incubated for 1 hour at 4° C. To finish incubation with the secondary antibody, sections were washed 3 times (5 min each) in cold TBS and then were incubated in an avidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector) for 2 hours at 4° C. Following the third incubation, sections were washed three times in cold TBS (5 min each), and once (10 min) in cold 50 mM Trizma buffer, pH 7.6. The section-containing carrousel was then transported to a Pyrex dish filled with 150 ml of 0.05% 3,3'-diaminobenzidine (DAB) in 50 mM tris buffer. Sections were immersed in this solution for 10 min at room temperature. Without being rinsed, sections were then transferred into a solution (150 ml) of DAB/tris buffer with 0.01% H_2O_2 to catalyze the reaction between DAB and hydrogen peroxidase, and 8% $NiCl_2$ to intensify the reaction product. This reaction was performed at room temperature and under constant agitation, and was interrupted by washing the sections 3 times (10 min each) with cold TBS.

Histology

Processed sections were transferred from the carrousel to a Pyrex Petri dish containing cold TBS and a few drops of gelatin mounting solution. The free floating sections were then mounted onto electrostatically-treated slides (SuperFrost Plus, Fisher Scientific) and were allowed to dry for at least one day. Sections were hydrated in distilled water (1 min) and gradually dehydrated in 70%, 90%, and 100% ethanol for 10 min. Slides were then cleared in xylene and coverslipped with Permount. The stimulating electrode tips coordinates were identified using landmarks located near by according to the Swanson (1992) sterotaxic atlas.

Data analysis

The distribution of FLIR was examined under a Leica microscope (Leitz DMRB). Quantitative analysis was conducted using a computarized image-analysis system (NIH Image 1.57). Counting of cell nuclei expressing FLIR was restricted to the following forebrain and midbrain structures: OT, prefrontal cortex (PFC), SEPT, nucleus accumbens (NA), caudate putamen (CP), MPOA, LPOA, BST, LHA, SI, paraventricular nucleus of the hypothalamus (PVN), AMY, posterior lateral hypothalamus (LHP), DMH, and VTA. All structures were defined according

to the Swanson stereotaxic atlas (1992), except for the PFC for which the stereotaxic atlas of Paxinos and Watson (1986) was employed. FLIR in each of these structures was measured as the number of labeled cells in a sampling area of 750 μm x 750 μm. Staining in each structure ipsilateral to the stimulating electrode was compared to staining in the homologous contralateral structure. Cell counts were made from three non-consecutive sections per structure for each animal.

Statistical analysis

The mean of cell counts of the three non-consecutive sections was computed for each structure in each animal for both stimulated and unstimulated hemispheres. Means were also computed in the two control subjects for each structure for both left and right hemisphere. Data derived from the 12 experimental subjects were organized in the following manner: Cell count means were divided into two groups according to the stimulation site (LH or VTA) and each group was subsequently divided according to the corresponding structure (15 structures). Cell count means in each structure were divided into two, depending on whether they were part of the ipsilateral or the contralateral hemisphere to the stimulation electrode (stimulated and unstimulated

hemispheres). To analyze these data a mixed-design 3-way ANOVA (CLR-Anova, Clear Lake Research Inc.) was used with stimulation site as the between subjects variable (2 levels: LH, VTA), and with structure (15 levels: OT, PFC, SEPT, NA, CP, MPOA, LPOA, BST, LHA, SI, PVN, AMY, LHP, DMH, VTA) and hemisphere (2 levels: ipsilateral, contralateral) as the two repeated measures. Test of simple main effects were applied to identify independent variables effects accounting for significant *F* values.

Results

The three-way ANOVA revealed a significant main effect for hemisphere [$F_{(1,10)} = 247.648$; $p = 0.00001$], stimulation site [$F_{(1,10)} = 8.68$; $p = 0.014$], and area [$F_{(1,140)} = 31.92$; $p = 0.00001$]. These results reflected the fact that there was more FLIR after LH than after VTA self-stimulation, more ipsilateral than contralateral to the stimulation electrode, and differing amounts as a function of area studied (see Appendix I for summary table)

Of greater importance, however, were the significant interactions. Although rewarding stimulation of both LH and VTA produced measurable FLIR in all of the areas examined, there were differences in the number of stained cells within some areas depending on the site of stimulation. These differences were reflected in the overall analysis as a significant interaction between stimulation site and area [$F_{(14,140)} = 4.273$; $p = 0.00001$]. Similarly, the number of cells exhibiting FLIR in the stimulated and unstimulated hemispheres varied as a function of the stimulation site. Differences in the degree of FLIR asymmetry were shown as a significant site x hemisphere interaction [$F_{(1,10)} = 5.206$; $p = 0.045$]. Finally, the analysis yielded a significant three-way interaction between stimulation site, area, and

hemisphere [$F_{(14,140)} = 10.777$; $p = 0.0001$]. The interaction between stimulation site and area differed as a function of hemisphere and the interaction between stimulation site and hemisphere differed as a function of area.

In order to localize the source of significance of the two- and three-way interactions, test of simple main effects were conducted. In the following sections, the results of these tests are presented for each significant interaction.

Stimulation site x hemisphere

Although there was more FLIR in the stimulated hemisphere regardless of stimulation site, there was a small but significant interaction between site of stimulation and hemisphere. To investigate whether the stimulated and unstimulated hemispheres displayed different levels of FLIR consequent to both LH and VTA self-stimulation, simple main effects of hemisphere were conducted. The results of these tests showed that both rewarding LH and VTA stimulation yielded asymmetrical patterns of FLIR between the two hemispheres. As depicted in figure 1, the average of Fos staining in all of the areas examined was significantly greater ipsilateral than contralateral to the stimulation electrode (see Appendix II for simple main effects analysis).

Effect of stimulation site ipsilateral and contralateral to the stimulation electrode

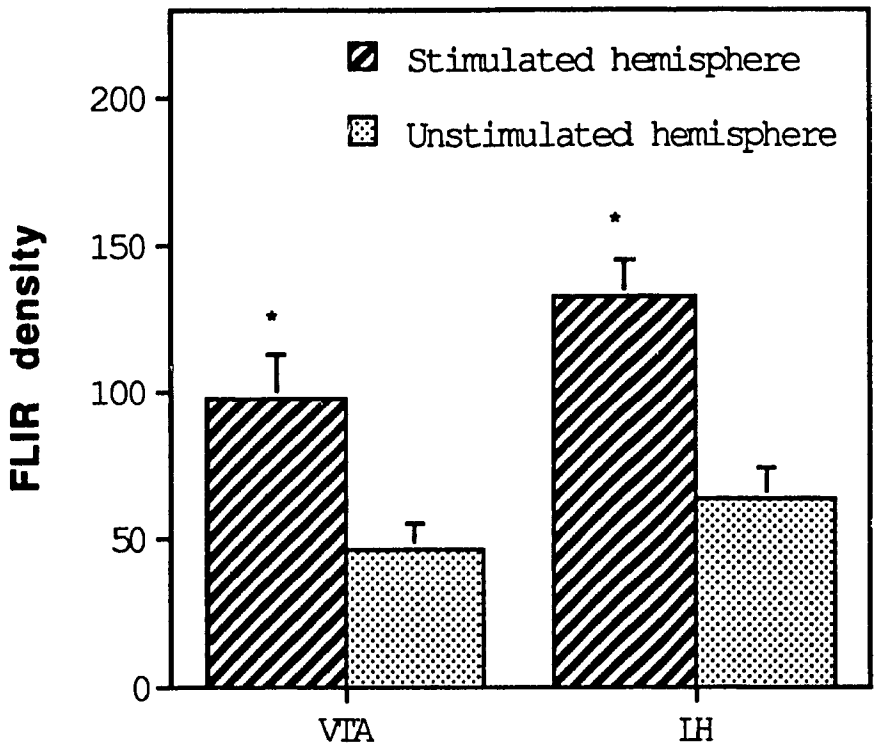


Figure 1. Mean number of Fos labeled cells (+ SEM) per 750 μm^2 in the stimulated and unstimulated hemispheres following 60 min of unilateral VTA or LH self-stimulation. FLIR density across the fifteen areas examined was averaged for each hemisphere. Asterisks represent differences significant at the 0.05 level.

Stimulation site x area

The significant interaction between stimulation site and area indicates that at least in some areas, the amount of FLIR was not the same following LH and VTA stimulation. To localize the areas that showed different Fos expression depending on the stimulation site, analysis of simple main effects of stimulation site were conducted. Figure 2 shows the mean number of labeled cells in the stimulated and unstimulated hemisphere for each area. Fos activation within the SEPT, NA, MPOA, LPOA, BST, SI, LHA, PVN and LHP was significantly greater following LH self-stimulation [ps < 0.05]. All the remaining areas, including the VTA, showed similar levels of FLIR following either LH or VTA self-stimulation (see Appendix III for simple main effects analysis).

Stimulation site x area x hemisphere

To identify the source of significance of the three-way interaction, two-way ANOVAs were conducted for each of the areas examined (see Appendix IV for summary tables). A significant main effect of hemisphere served as an indication of asymmetrical staining for a particular area. A significant main effect of stimulation site provided

Distribution of Fos-like immunoreactivity

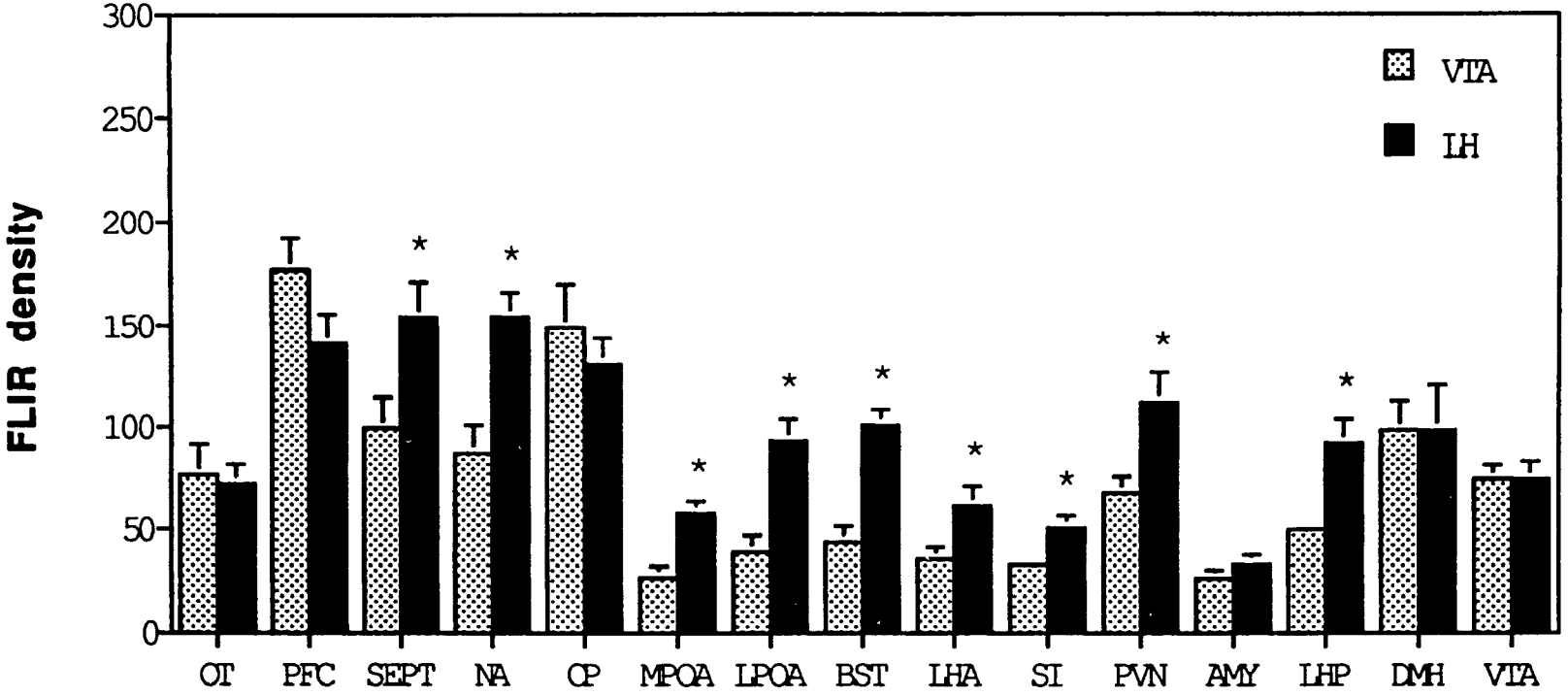


Figure 2. Mean number of Fos labeled cells (+ SEM) per 750 μm^2 in each of the examined areas following 60 min of unilateral VTA or LH self-stimulation (n=6 for each group). FLIR density in the stimulated and unstimulated hemispheres was averaged for each area. Asterisks represent differences significant at the 0.05 level.

information about differences in FLIR after LH and VTA for that particular area (Fig. 2). A significant interaction between site of stimulation and hemisphere suggested that the degree of FLIR asymmetry between the two hemispheres for that particular area is not the same after LH and VTA self-stimulation.

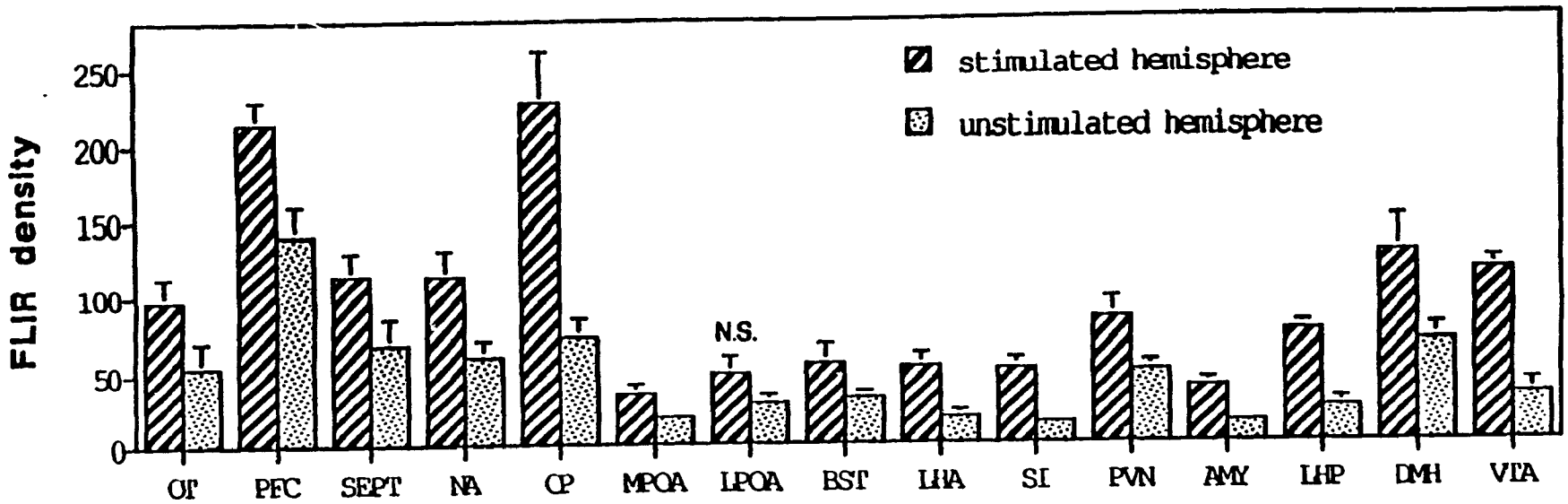
The main effect of hemisphere was significant for all of the areas assessed showing that a certain degree of FLIR asymmetry was produced after self-stimulation. Indeed, there were several areas in which the asymmetry in Fos staining was very similar after LH and VTA self-stimulation. Asymmetrical activation of Fos within the NA, PVN, AMY, DMH and VTA was not significantly different after LH and VTA self-stimulation (Fig. 3, 6e, 6f, 7e, 7f, 8e, 8f, 9e, 9f) as reflected by a non-significant stimulation site x hemisphere interaction in the outcome of the two-way ANOVAs conducted for each of these structures [ps > 0.05].

In another set of areas, however, the asymmetrical expression of Fos was not the same after LH and VTA self-stimulation. As reflected by significant two-way interactions, asymmetry in FLIR between the stimulated and unstimulated hemisphere differed significantly with the site

Figure 3. **A.** Mean number of Fos labeled cells (+ SEM) per 750 μm ^{2} in the stimulated and unstimulated hemisphere following 60 min of unilateral VTA self-stimulation (n=6). **B.** Mean number of Fos labeled cells (+ SEM) per 750 μm ^{2} in the stimulated and unstimulated hemisphere following 60 min of unilateral LH self-stimulation (n=6). N.S. represents no significant difference ($p>.05$).

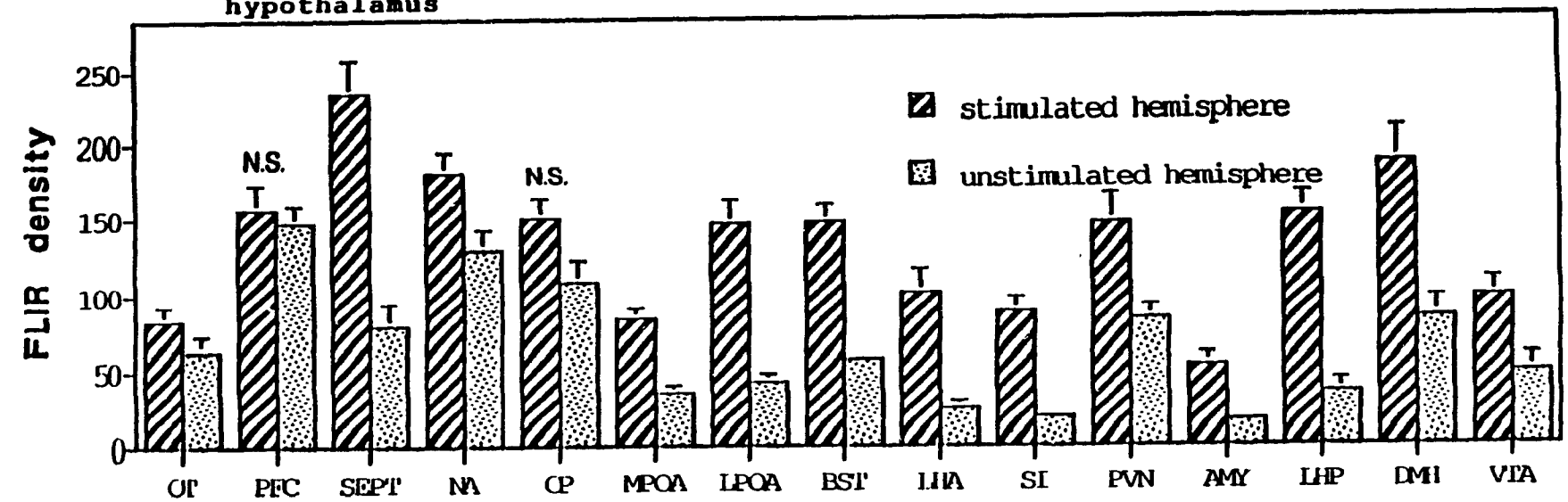
A.

Fos expression following self-stimulation of the ventral tegmental area



B.

Fos expression following self-stimulation of the lateral hypothalamus



of stimulation in the OT, PFC, CP, SEPT, MPOA, LPOA, SI, BST, LHA, and LHP [ps $\leq$ 0.05]. As shown in figure 3, the OT, PFC and CP showed a more pronounced asymmetrical FLIR following VTA self-stimulation than LH self-stimulation (Fig. 8c, 8d 9c, 9d). In fact, there were no significant differences between stimulated and unstimulated hemispheres in the PFC and CP after LH self-stimulation (Fig. 3b). On the other hand, asymmetry of staining was more pronounced after LH self-stimulation within the SEPT, MPOA, LPOA, SI, BST, LHA, and LH, (Fig. 3, 6, 7, 8a, 8b, 9a, 9b). In the case of the LPOA there was no significant difference between the two hemispheres following VTA electrical stimulation (Fig. 3a) (see Appendix V for simple main effects analysis).

Control subjects

Visual inspection of sections from the unstimulated control brains revealed differences in fos expression between the naive and trained subjects. Sparse labeling was seen in the brain from the naive subject. Although, Fos expression in the brain of the trained control was negligible in most of the structures inspected, bilaterally labeled cells within the SEPT, MPOA, LPOA, and AMY were observed and counted (Fig. 4). The presence of labeled nuclei within those

Areas exhibiting Fos expression in the trained control subject

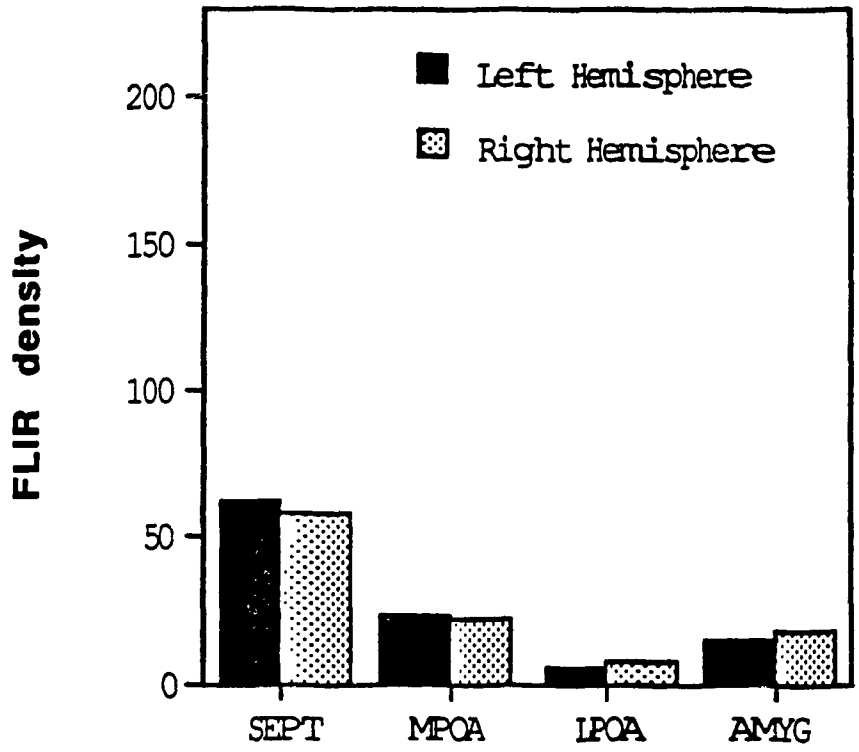


Figure 4. Number of Fos labeled cells per 750 μm^2 in the sections obtained from the trained control subject (n=1). The areas that are not shown in the graph exhibited none or very low levels of FLIR.

areas suggests that the manipulations undertaken prior to perfusion were sufficient to cause Fos activation.

Identification of Fos activation in the trained control brain posed the question of whether FLIR observed in similar areas in the stimulated hemisphere of both LH an VTA stimulated brains could have resulted from manipulating the rats (handling, place them in the stimulating boxes, etc.) and/or conditioning effects rather than from the stimulation itself. For this reason, Fos levels in the two control brains were compared to those measured in the stimulated hemisphere of the two experimental groups.

In figure 5 the number of labeled cells found in the brains of the control and experimental subjects are plotted for each area examined. FLIR for each control subject represents the average of the left and right hemispheres. FLIR for the experimental subjects represents the mean of each group (n=6). Although differences in FLIR were not statistically assessed, expression of Fos in the two control brains appeared to be less than the expression of Fos in the stimulated hemisphere following LH self-stimulation. The same seemed to be true for the VTA group, except for the MPOA in which the difference in FLIR between the trained control

Expression of Fos in the control and experimental subjects

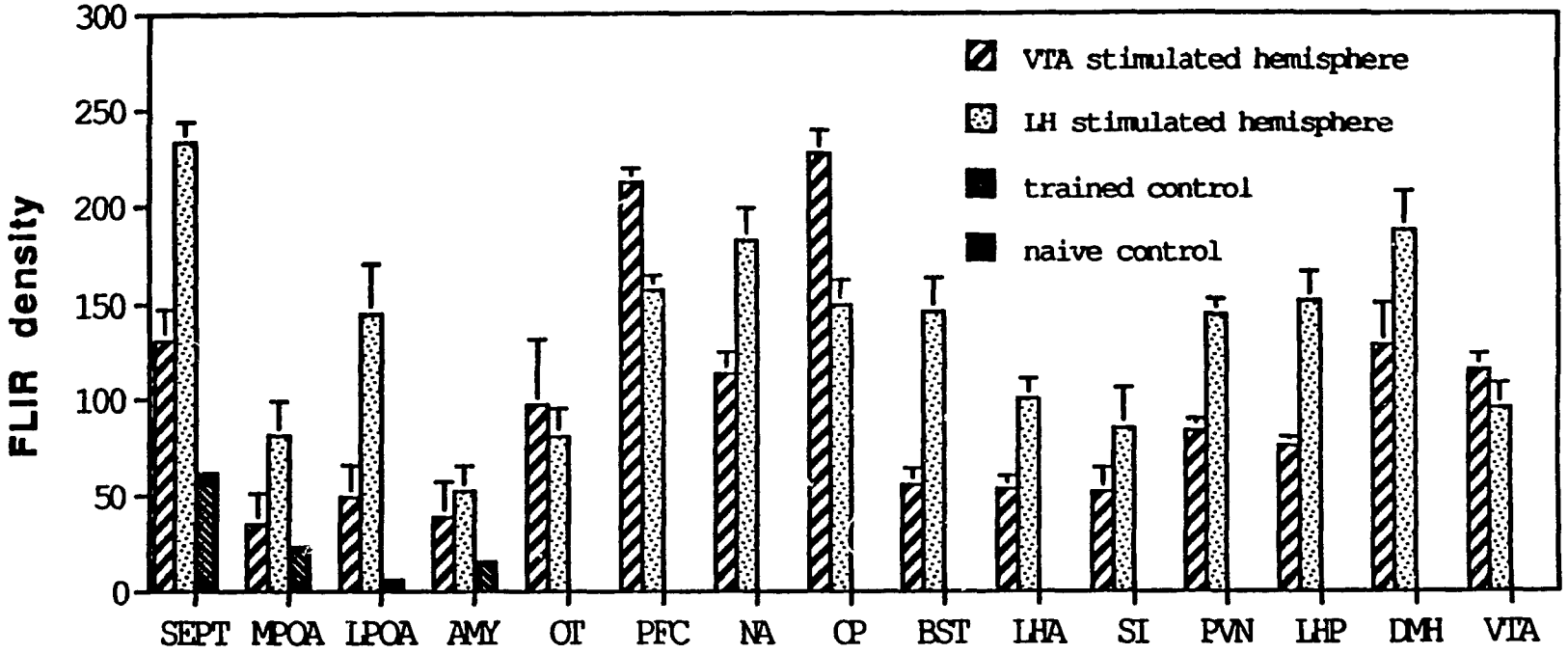


Figure 5. Mean number of Fos labeled cells (+ SEM) per 750 μm^2 in the stimulated hemisphere following LH and VTA self-stimulation (n=6 for each group). FLIR density in the trained and naive control subjects (n=1) represents the average number of labeled cells per 750 μm^2 in the left and right hemispheres.

and the VTA stimulated group was not apparent. None of these differences, however, was tested for significance.

During the quantitative analysis, Fos staining was noticed in some brains in the periaqueductal gray, dorsal raphe, pedunculopontine nucleus (PPN) and claustrum, following both VTA and LH self-stimulation. However, none of these areas was examined in a quantitative manner.

It is important to mention that the subject that displayed a strong motor effect contralateral to the stimulation site showed disproportional high levels of Fos within the CP ipsilateral to the stimulation electrode. To prevent this outlier from influencing the overall mean, FLIR within the CP for that particular subject was not included in the analysis and was replaced by the group mean.

Electrode tip locations for the VTA and LH stimulation electrodes are shown in figure 10 and figure 11 respectively. Histological reconstructions were made onto tracings of coronal plates from the Swanson atlas (1992, 1992-4).

Figure 6. Representative digitized images showing FLIR consequent to VTA self-stimulation in the MPOA, LPOA, BST (A), SI (C, D), LHA, and PVN (E, F). FLIR in the stimulated hemisphere (B, D, F) was more pronounced than in the unstimulated one (A, C, E). Scale bar = 200 μm.

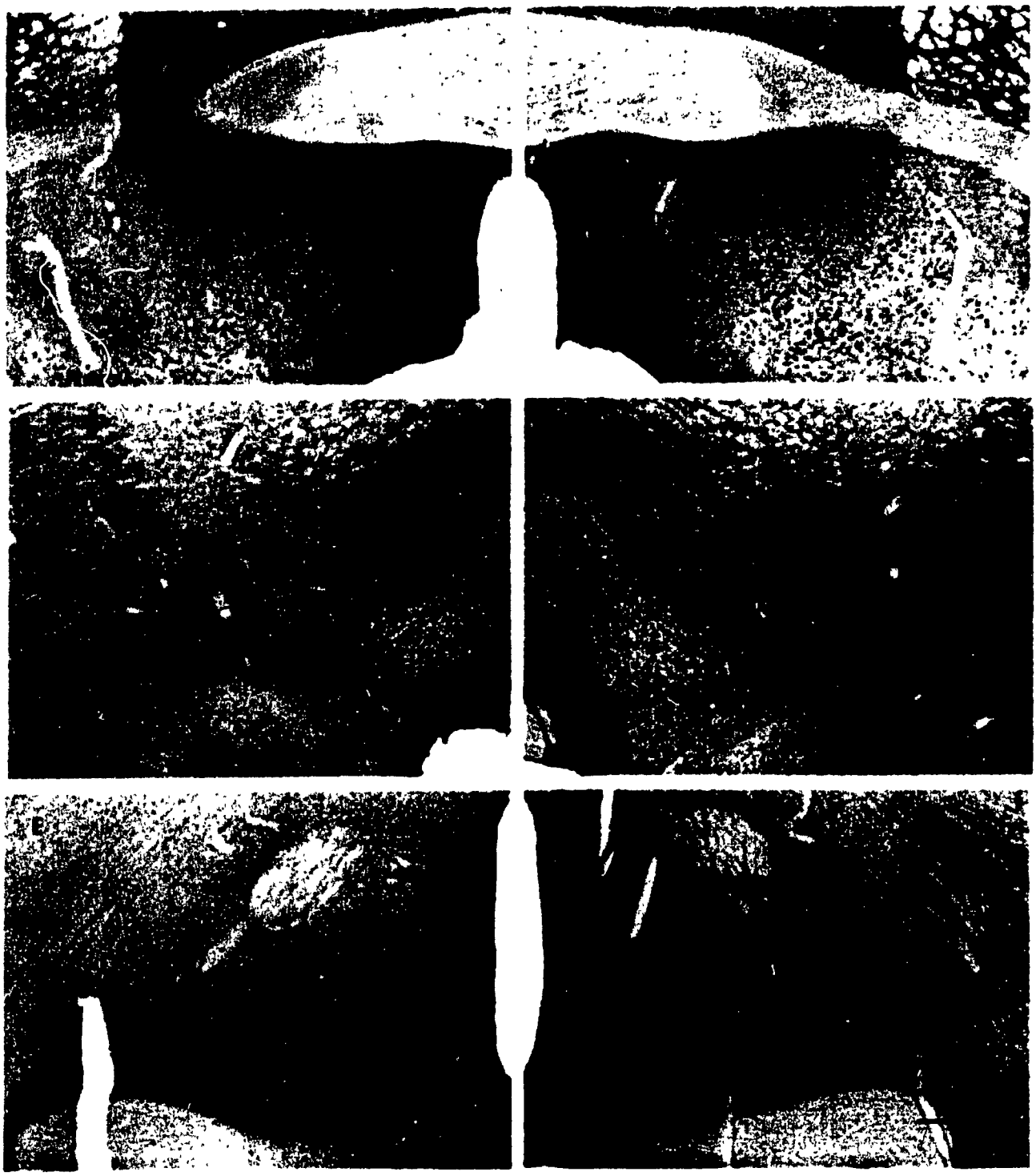


Figure 7. Representative digitized images showing FLIR consequent to LH self-stimulation in the MPOA, LPOA, BST (A), SI (C, D), LHA, and PVN (E, F). FLIR in the stimulated hemisphere (B, D, F) was more pronounced than in the unstimulated one (A, C, E). Scale bar = 200 μm.

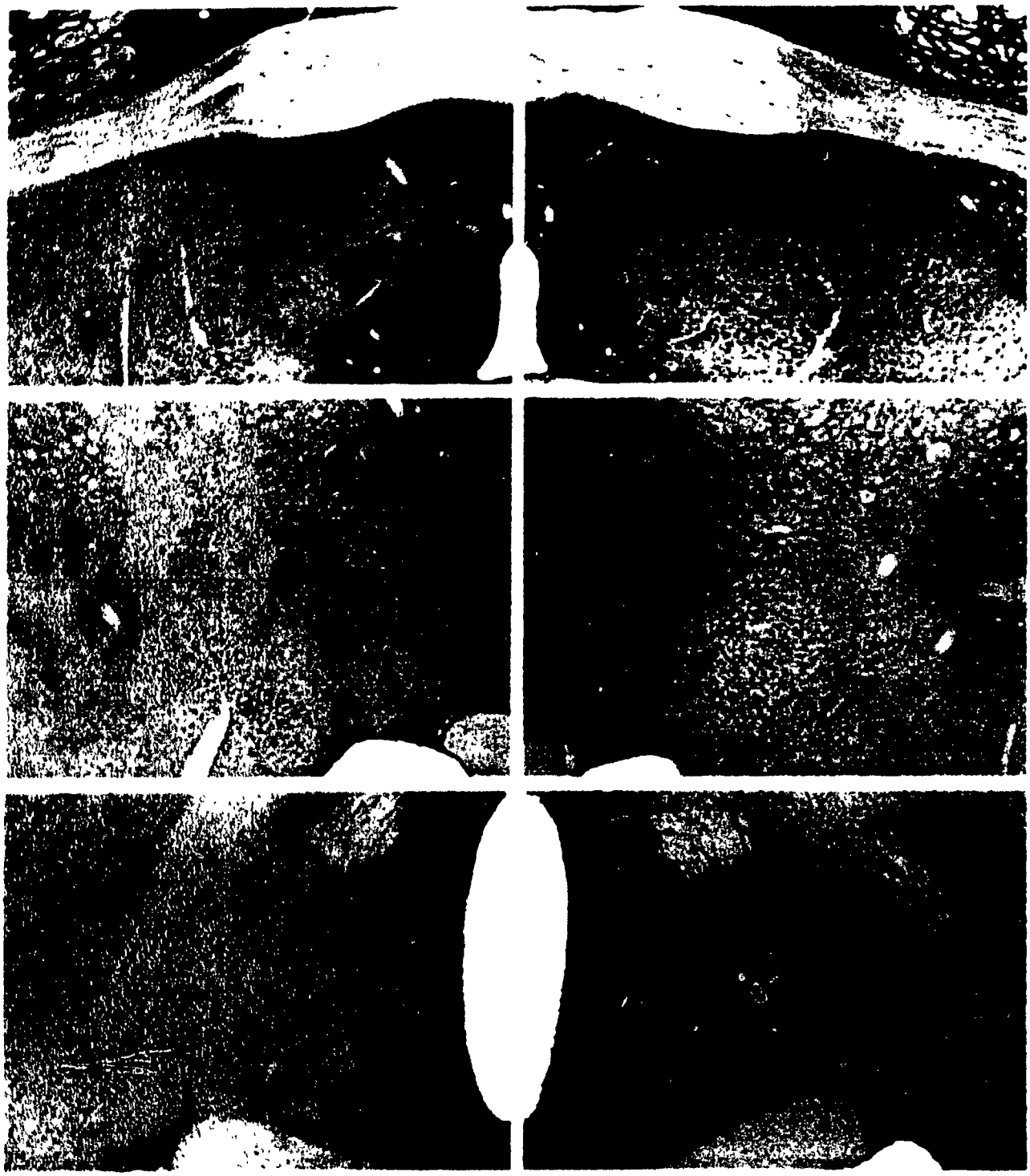


Figure 8. Representative digitized images showing FLIR consequent to VTA self-stimulation in the SEPT (A), CP (C, D), and VTA (E, F). FLIR in the stimulated hemisphere (B, D, F) was more pronounced than in the unstimulated one (A, C, E). Scale bar = 100 μm.

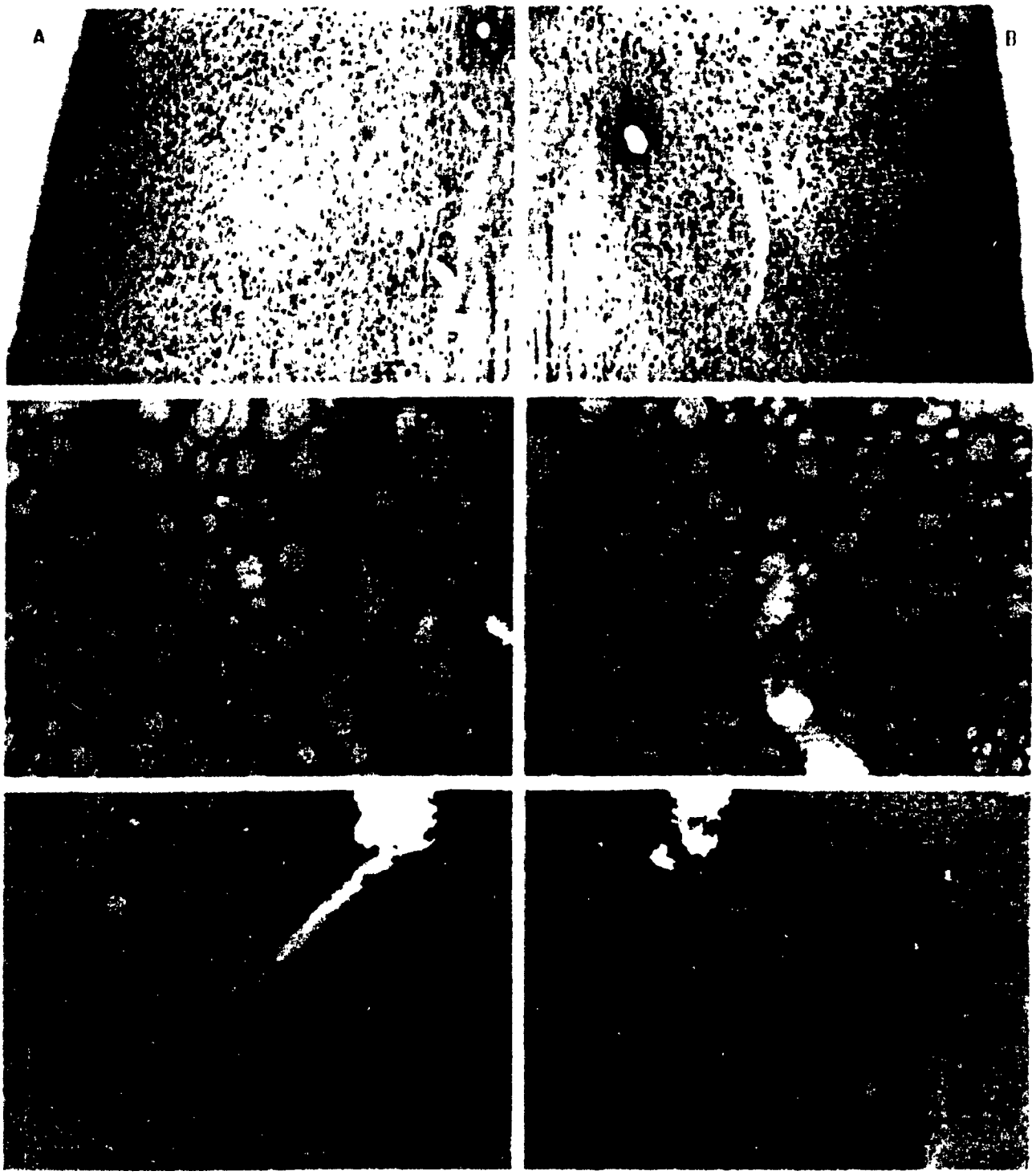

A
B

Figure 9. Representative digitized images showing FLIR consequent to LH self-stimulation in the SEPT (A), CP (C, D), and VTA (E, F). FLIR in the stimulated hemisphere (B, D, F) was more pronounced than in the unstimulated one (A, C, E). Scale bar = 100 μm.

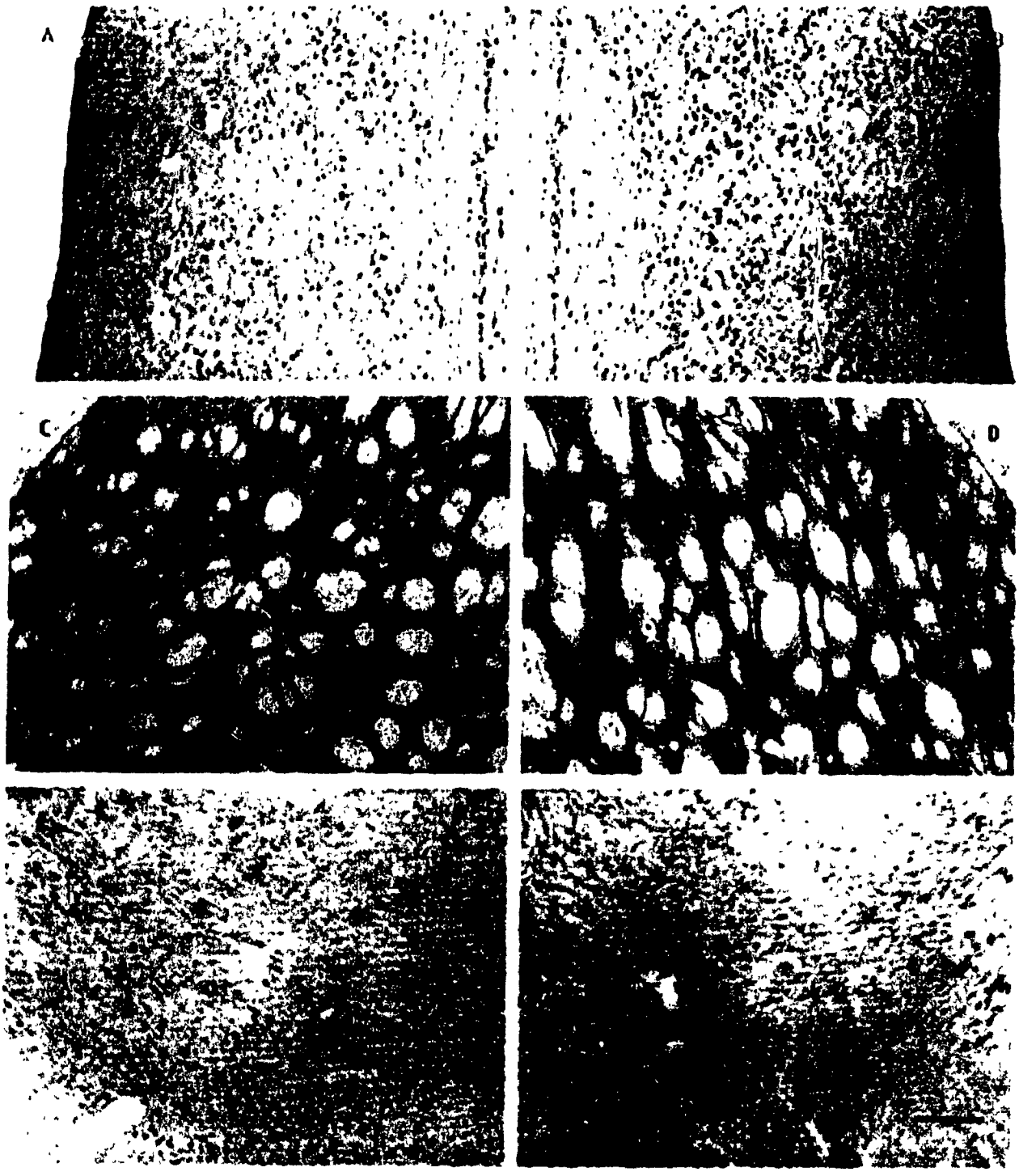

A
C
D

Figure 10. VTA stimulation electrodes for the 6 subjects. Electrode tips are represented as stars and were reconstructed onto tracings of coronal plates from the Swanson atlas (Swanson, 1992, 1992-4)

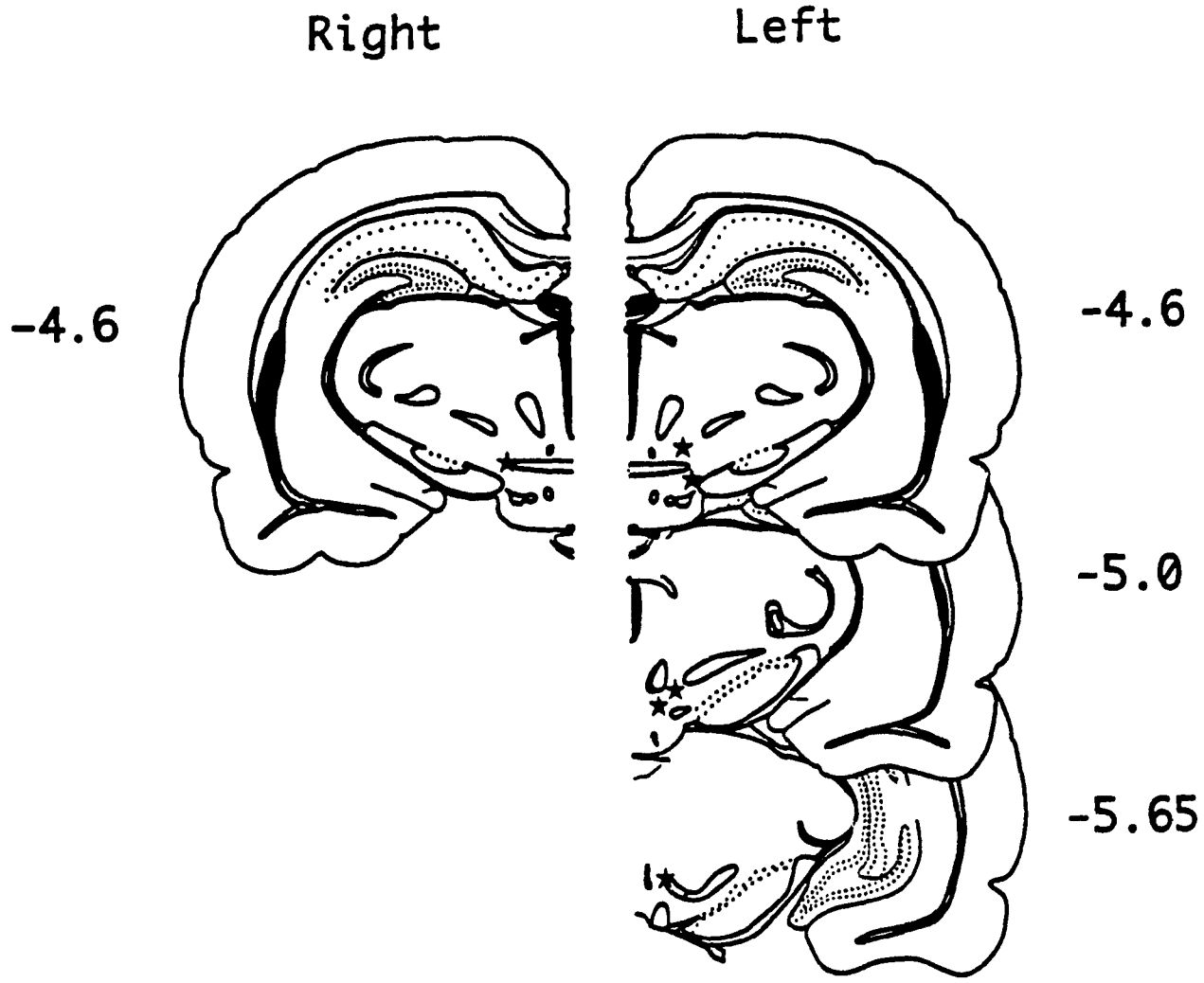

Right
Left
-4.6
-4.6
-5.0
-5.65

Figure 11. LH stimulation electrodes for the 6 subjects. Electrode tips are represented as stars and were reconstructed onto tracings of coronal plates from the Swanson atlas (Swanson, 1992, 1992-4)

Right Left

Discussion

Collision studies (Shizgal et al., 1980, Murray & Shizgal, in press,a) have demonstrated that some of the fibers of the first-stage neurons are continuous between the LH and VTA levels of the MFB. Although the somata that give rise to these fibers have not yet been identified, there is evidence suggesting that some of them are localized in the basal forebrain. Using electrophysiological techniques, neurons activated antidromically by LH and VTA stimulation with physiological properties that match those of the first-stage substrate have been found in basal forebrain regions (Rompré & Shizgal 1986; Shizgal et al., 1989; Murray and Shizgal, in press,b). Lesioning some of these basal forebrain structures, including the LHA and SI, attenuates the rewarding effects of both LH and VTA stimulation (Murray and Shizgal, 1991, 1995c; Arvanitogiannis et al., in press). Fos expression has been found ipsilateral to LH self-stimulation electrodes in cell nuclei within these basal forebrain regions (Arvanitogiannis et al., submitted for publication). The purpose of the present study was to determine the extent to which rewarding stimulation of either LH or VTA induces expression in the same basal forebrain nuclei. It was found that neurons within the LHA, SI, and BST showed Fos induction ipsilateral to the electrode after

stimulation of either the LH or the VTA. Other structures, such as the LPOA were also activated, but the distribution of FLIR after VTA stimulation was bilateral. These findings show that basal forebrain nuclei already implicated in BSR are, indeed, activated by rewarding stimulation of both the LH and VTA. Nonetheless, it should be kept in mind that the method employed to visualize activated cells cannot distinguish first-stage cells from other reward-relevant neurons or from activated neurons that are not involved in BSR.

Interhemispheric distribution of Fos expression following LH and VTA self-stimulation

Because most projections in the MFB are unilateral (Nieuwenhuys et al., 1982; but see Miliaressis & Malette, 1994) it is reasonable to suggest that most of the FLIR resulting from unilateral MFB stimulation should be seen in the stimulated hemisphere. However, it is possible to obtain FLIR in the unstimulated hemisphere either because a small proportion of stimulated fibers cross to the contralateral side or because some efferents of MFB fibers send forth bilateral projections. In fact, Fos activation has been observed at least two synapses from the site of the stimulation (Sagar, Sharp and Curran; 1988).

In the next few paragraphs the distribution of FLIR in the stimulated and unstimulated hemispheres will be discussed. To facilitate data presentation the structures analyzed for Fos staining are divided into three main groups on the basis of their anterior-posterior location in the brain. The first group (Group A) comprises the more rostral of the structures analyzed, which include the OT and the PFC and also the dorsal structures of the basal forebrain (SEPT, NA, and CP). All of the structures in Group A receive dopaminergic projections (Fallon & Moore, 1978). The second group (Group B) consists of basal forebrain structures (MPO, LPO, BST, SI and AMY), and the third one (Group C) is composed of hypothalamic areas (LHA, PVN, LHP, and DMH) (Phillipson, 1979, De Olmos, Alheid & Beltramino, 1985) and the VTA.

FLIR in Group A

All of the structures analyzed exhibited FLIR after LH or VTA stimulation. Interhemispheric differences detected within the structures examined varied depending upon the stimulation site. As figure 3a illustrates, VTA self-stimulation yielded prominent asymmetrical staining within the following rostral regions: the OT, PFC, CP, NA and the SEPT. Given that these structures receive abundant

dopaminergic innervation (Fallon & Moore, 1978) and that dopamine activation induces Fos expression in the striatum (Morelli et al., 1992), one might expect that part of the observed immunoreactivity resulted from the direct activation of VTA dopaminergic cells. However, it has been shown that due to their electrophysiological properties, few dopaminergic cells are likely to be directly activated by the stimulation parameters commonly used in BSR studies (Yeomans, Maidment and Bunney, 1988; Anderson, Fatigati & Rompré. 1995).

Asymmetrical labeling in the aforementioned regions after VTA stimulation could be due to certain factors that do not necessarily include direct dopaminergic activation. First of all, dopaminergic cells in the VTA can be activated transsynaptically by descending MFB fibers, and in turn transsynaptically activate forebrain neurons.

Secondly, not all of the neurons in the VTA are dopaminergic. Hence, neurons containing other neurochemicals may also contribute to the ascending projections and, therefore, to the expression of Fos. For instance, Van Bockstaele & Pickel (1995) have recently demonstrated that gamma-aminobutyric acid (GABA)-containing cells within the VTA extend to the NA. While the purpose of this example is

to confirm the existence of non dopaminergic cells within the VTA, it is worth mentioning that despite GABA's inhibitory action, its activation can result in Fos expression in the innervated area through disinhibition of other neurons. In as much as non-dopaminergic neurons within the VTA are activated by the stimulation electrode, rostral regions receiving outputs from these neurons are likely to exhibit FLIR.

A third factor that could account for the ipsilateral increase in FLIR within the most rostral regions, following VTA self-stimulation, is the direct activation of their descending fibers. Substantial anatomical evidence has shown that neurons within the OT, PFC, NA and SEPT give rise to fibers that extend through the MFB to the VTA (Phillipson, 1979; Nieuwenhuys et al., 1982). Antidromic activation of these neurons through the VTA stimulation electrode could well result in ipsilateral expression of Fos. The contribution of CP projections to the MFB, however, is controversial (Nieuwenhuys et al, 1982). Therefore, antidromic activation of MFB fibers may not be an adequate explanation for the asymmetrical FLIR within this structure. Nevertheless, it is well established that the CP and the substantia nigra (SN) communicate with each other through reciprocal projections (Fallon & Moore, 1978; Heimer, Alheid

& Zaborszky, 1985). Perhaps then, the VTA stimulating electrode antidromically activated the descending component of these projections.

Contrary to the asymmetrical FLIR observed in rostrally located structures after VTA stimulation, bilateral staining was obtained with LH stimulation within the OT, PFC, and CP (Fig. 3b). The absence of asymmetrical staining in these regions after LH stimulation is rather puzzling, especially when one considers that LH stimulation results in the transsynaptic activation of dopamine neurons within the VTA.

The different FLIR distribution seen in rostral areas after LH and VTA stimulation may have arisen simply from their different anatomical connections. Thus, a particular explanation offered to account, say, for unilateral expression of Fos at one site, may or may not hold true for stimulation at the other site. For example, the fact that there exist reciprocal projections between the SN and the CP may explain the ipsilateral expression of Fos after VTA stimulation. However, as already discussed, these projections may not be part of the MFB and, thus, would not be expected to be activated by LH stimulation.

At any rate, FLIR within the CP should be interpreted with caution. As depicted in figure 3, FLIR within this region varied considerably between subjects. As was mentioned in the results section, one of the LH stimulated subjects showed disproportional high levels of Fos. Interestingly, this rat exhibited a strong motor effect contralateral to the stimulation site. Given that the CP is implicated in locomotor behavior and in particular in motor stereotypies (Domesick, 1981), it may be hypothesized that FLIR was induced in this area not only by the electrical stimulation, but also by the strong activation of motor circuits.

As was the case after VTA self-stimulation, asymmetrical Fos labeling after LH self-stimulation was observed within the SEPT and the NA. Given that the two areas send descending fibers through the MFB that transverse the LH and the VTA it is reasonable to assume that at least some of the activated cells displaying Fos labeling were antidromically activated by either stimulation electrodes.

On the basis of the asymmetrical Fos expression found within the SEPT and NA following LH and VTA self-stimulation, cells in the SEPT and NA could arguably form a part of the first-stage substrate. However, when the role of the SEPT in

BSR was assessed by means of knife cuts no meaningful changes in the rewarding impact of LH stimulation were noticed (Waraczynski, 1988). Similarly, bilateral NMDA lesions of the NA did not attenuate LH stimulation reward. Modest decreases in reward effectiveness were seen in only one subject (Johnson & Stellar, 1994). The ability of lesions of the SEPT or NA to affect VTA self-stimulation has not been tested.

As shown in figure 3, LH and VTA self-stimulation led to Fos expression in the unstimulated hemisphere. Fos staining in contralateral rostral regions could, at least in part, be explained by a model of BSR-relevant circuitry proposed by Yeomans (1993). He argues that the first-stage neurons send descending projections that synapse to cholinergic cells within the PPN. These cells in turn, project *bilaterally* to the VTA where they activate dopaminergic neurons. Assuming the accuracy of this model, bilateral activation of dopaminergic cells contribute to Fos staining in contralateral rostral regions. However, a problem with Yeoman's model is that according to it, MFB stimulation of either hemisphere should activate VTA dopamine neurons on both sides of the brain. Thus, unilateral interference with the dopaminergic neurotransmission would affect self-stimulation bilaterally. However, there are several reports

in which unilateral interference with the mesolimbic dopamine system affects ipsilateral but not contralateral MFB self-stimulation (Fibiger, LePiane, Jakubovic & Phillips, 1987).

FLIR in Group B

Extensive evidence from anatomical studies shows that basal forebrain regions send projections through the MFB to the LH and VTA (Nieuwenhuys et al., 1982; Phillipson, 1979; Swanson, 1979). Data presented here show a difference in FLIR between the stimulated and unstimulated hemispheres in the MPOA, BST, SI, and AMY for stimulation of both the LH and the VTA (Fig. 3). Asymmetrical staining of the LPOA reached significance only in the case of LH self-stimulation. The interhemispheric differences in FLIR were considerably larger after LH self-stimulation. The simplest explanation for the difference in the degree of asymmetrical staining following LH and VTA stimulation is that the density of the efferent fibers from basal forebrain nuclei may be higher at the level of the LH than the VTA. Electrophysiological evidence showing that basal forebrain neurons are antidromically driven by LH and VTA stimulation (Rompré and Shizgal, 1986; Shizgal et al., 1989, Murray & Shizgal, 1995 in pressb), suggests that many of the labeled cells were directly activated by the electrode.

The possible relevance of the MPOA in BSR is suggested by the massive descending projections from this area to the LH and VTA (Simerly & Swanson, 1988), by single-unit recordings performed in this area (Shizgal et al., 1989), and by the previous Fos study (Arvanitogiannis et al., submitted for publication). Recently, Arvanitogiannis, Riscaldino and Shizgal (1995) have reported that NMDA lesions of the MPOA do not appreciably reduce the reward efficacy of either LH or VTA stimulation. Although preliminary, this evidence challenges the hypothesis that the MPOA contains neurons important to MFB self-stimulation. Electrolytic lesions to the AMY have also failed to reduce the rewarding impact of both LH and VTA stimulation (Waraczynski et al., 1990).

The LPOA and the SI are two basal forebrain nuclei that have been strongly implicated in BSR. Recently, selective cell body lesions encompassing the LPOA and in some cases the SI have resulted in long lasting, large decreases in reward effectiveness for electrical stimulation of the LH (Arvanitogiannis et al., in press). Electrophysiological recordings of LPOA (Rompré & Shizgal, 1986; Shizgal et al., 1989) and SI (Murray & Shizgal, 1995 in press,b) neurons antidromically activated by LH an/or VTA stimulation revealed that the refractory period and conduction velocity estimates for these neurons overlap the psychophysically derived

estimates for MFB reward neurons. The present study extends these findings and provides a further link between neurons in the LPOA and SI and the reward relevant substrate.

This having been said, a closer inspection of the above-mentioned lesion study reveals a paucity of cases where lesions reduced the reward effectiveness of VTA stimulation. In an analogous manner, asymmetrical Fos expression within the LPOA was not observed after VTA self-stimulation. Given the evidence from anatomical studies that a common bundle of MFB fibers extends between the LH and the VTA, this may seem puzzling. However, it should be kept in mind that not all the projections from the LPOA to the LH reach the VTA, but only the ones that arise in the posterolateral aspects.

Fos expression in the SI was asymmetrical after both LH and VTA self-stimulation. That a change in the reward effectiveness of VTA stimulation was not observed in the excitotoxic lesion study does not rule out the contribution of the SI to MFB self-stimulation. To date there has been no lesion study specifically aimed at the SI. In the excitotoxic lesion study the focus was on the destruction of the LPOA and the SI was damaged only as an uncontrolled consequence of the spread of the lesion and occurred only in two animals. However, Murray & Shizgal (in press,c) found

substantial increases in LH and VTA self-stimulation thresholds after electrolytic lesions that in at least one subject damaged a significant portion of the SI. Future lesion studies should be targeted at the SI.

Another basal forebrain structure whose involvement in BSR warrants further investigation is the BST. Following either LH or VTA self-stimulation asymmetrical labeling was seen within the BST. However, little has been done to assess the role, if any, this structure plays in BSR.

I have discussed the distribution of FLIR in basal forebrain structures in terms of antidromic activation of their descending fibers. Of course, a contribution from transsynaptically activated cells within these areas cannot be ignored. Anatomical evidence shows that ascending projections from the LH and VTA terminate in basal forebrain nuclei. Moreover, basal forebrain structures are connected with one another, thus activation of one may lead to activation of others.

FLIR in Group C

All of the structures that were analyzed in Group C (LHA, PVN, DMH, LHP, and VTA) showed asymmetrical FLIR following stimulation of both the LH and the VTA (Fig. 3). The role of some of these structures in BSR has already been assessed. Waraczynski et al., (1992) showed by means of electrolytic lesions that neurons arising in, terminating in, or projecting through the DMH are not important in self-stimulation of the rostral VTA. Similarly, excitotoxic lesions of the LHP produced only minimal and temporary LH stimulation reward deficits (Stellar et al., 1991).

On the other hand, various lesion studies have demonstrated that the LHA may significantly contribute to MFB reward. In the NMDA lesion study of Arvanitogiannis et al. (in press) the damage associated with the large effects invariably included the LHA. Moreover, two electrolytic lesion studies by Murray & Shizgal (1991, in press,c) aimed at the LHA obtained, in several cases, long-lasting and substantial increases in both LH and VTA self-stimulation thresholds. Taken together with the results of lesion studies, the present data reinforce the notion that the LHA may contain at least some of the first-stage cells.

According to the results of this study, the PVN is a region worth of further investigation. Of course, Fos expression is not necessarily or exclusively restricted to reward relevant cells. The PVN is known to play a role in multiple regulatory physiological functions including stress (Rivier & Plotsky, 1986) .

It was not surprising that asymmetrical expression of Fos was observed within the VTA following stimulation of this site. Furthermore, ipsilateral increases in Fos staining in the VTA after LH self-stimulation can be explained either by the transsynaptic activation of dopaminergic cells or by antidromic activation, primarily of non dopaminergic neurons.

Overall expression of Fos following LH and VTA self-stimulation

Fos-labeled cells were found within all the areas examined following both LH and VTA self-stimulation. However, the number and distribution within most structures differed after stimulation at each site. As depicted in figure 2, the higher levels of Fos expression following LH stimulation were mainly localized in basal forebrain regions (MPOA, LPOA, BST, SI and LHA), although more rostral (SEPT

and NA) or more caudal (LHP and PVN) structures showed large numbers as well.

The quantitative difference in Fos staining found between the two stimulation sites can be attributed to the fact that some of the fibers that arise in the forebrain terminate in regions anterior to the VTA. Thus, neurons whose outputs project through the LH, but not the VTA would express Fos only when the electrode is in the LH. In the case of the LPOA, for example, only the posterolateral part sends axons to the VTA. Accordingly, in the present study, FLIR after VTA stimulation was detected in the more caudal aspects of the LPOA whereas FLIR after LH stimulation was seen throughout the entire structure.

Caveats of the present study

The interpretation of the results presented here is limited by several factors. First, since the MFB consists of myriad fibers that originate in different nuclei, Fos activation will be seen in many regions that are not necessarily implicated in BSR. The technique employed to visualize activated cells cannot discriminate between reward relevant neurons and other neurons that do not contribute to MFB self-stimulation. Thus, Fos immunohistochemistry alone

is not enough to pinpoint the first-stage neurons. Second, the fact that the expression of Fos can result from antidromic as well as transsynaptic activation does not permit us to distinguish cells directly activated by the electrode that are part of the first-stage neurons from cells that are transsynaptically activated and play a secondary role in BSR or from cells that are activated transsynaptically and do not play a role in BSR. Third, it is not clear that Fos can be expressed in every activated cell. Neurons in some brain regions that are known to become activated after certain manipulations do not show FLIR. For example, tactile whisker sensory stimulation does not induce FLIR in any of the whisker-related pathways (Sharp, Hisanaga & Sagar, 1993). Perhaps some cells lack the required biochemical machinery necessary to induce *c-fos*. Thus, one cannot conclude that the structures exhibiting FLIR are the only ones activated during LH and VTA self-stimulation.

Within this context, the results presented here are best considered together with electrophysiological and lesion data. The immunohistochemical technique serves solely as the basis for identifying regions asymmetrically activated after both LH and VTA self-stimulation. These regions could be worthy of further investigation. If some regions that expressed asymmetrical FLIR have also been shown to be

implicated in BSR by means of electrophysiological and lesion experiments, then it is possible to argue that such structures could be involved in the rewarding effects of self-stimulation. Accordingly, the SI and LHA seem to be strong candidates to contain at least part of the first-stage substrate for the rewarding effects of both LH and VTA stimulation. The LPOA seems to have candidate cells relevant to the rewarding effects of LH stimulation. Structures such as the BST merit further investigation.

Fos and other metabolic markers

In order to visualize areas where there is a high density of activated tissue, other metabolic markers have already been used in prior experiments on MFB self-stimulation. Gallistel, Gomita, Yadin and Campbell (1985) used 2-DG to determine which forebrain and diencephalic areas show metabolic alterations in response to 45 min of LH and VTA self-stimulation. High uptake of the metabolic marker was observed in some forebrain regions including the SEPT, MPOA, BST and LPOA, and in more caudal regions such as the VTA. Increases in glucose utilization during LH self-stimulation have also been reported in regions such as the NA and OT both ipsilateral and contralateral to the site of the stimulation (Porrino, Huston-Lyons, Bain, Sokoloff, and

Kornetsky, 1990). Cytochrome oxidase (CO), an endogenous mitochondrial enzyme that is linked to metabolic processes, has also been used to reveal activity-related changes resulting from rewarding LH stimulation. Asymmetrical staining has been observed by means of this technique in several structures ipsilateral to the stimulating electrode, such as the SEPT and the NA (Bielajew, 1991).

Although the use of Fos immunohistochemistry as a marker of neuronal activity is not free of difficulties of interpretation, its use has significant advantages over other imaging methods. As mentioned in the introduction, Fos immunohistochemistry stains the nucleus, making it possible to visualize the soma of the activated neurons. In contrast, 2-DG autoradiography lacks this cellular resolution. Furthermore, the use of 2-DG and CO poses the problem of whether the increases in metabolic activity observed following MFB self-stimulation occur in neurons intrinsic to a brain region or in terminals of fibers afferent to that region. Given that the main goal of many BSR experiments is to localize the neurons that belong to the first-stage substrate, Fos immunohistochemistry is better suited to delimit the areas for a concerted search. Another advantage of Fos immunohistochemistry that arises from the nuclear location of the Fos protein is that it can be combined with

labeling methods such as axonal markers and cytoplasmic proteins associated with particular neurotransmitter systems. Of utmost interest in BSR experiments is to trace the location of first-stage neurons and to find out the neurotransmitter(s) released when these neurons are activated. By combining Fos immunohistochemistry with other labels or labeling methods one could potentially answer such questions.

Future studies

The main purpose of this study was to visualize neurons activated by *both* LH and VTA self-stimulation in order to delimit the search for the first-stage substrate responsible for the rewarding effects of MFB stimulation. Although Fos immunohistochemistry offers several advantages over other imaging techniques, it does not provide the means by which one could distinguish antidromically activated neurons from those transsynaptically activated. However, the fact that Fos accumulates in the cell nucleus without significantly marking the cytoplasm permits the use of Fos immunohistochemistry in combination with tracing techniques that stain the cytoplasm. This combination could prove powerful enough to further restrict the search for the first-stage substrate.

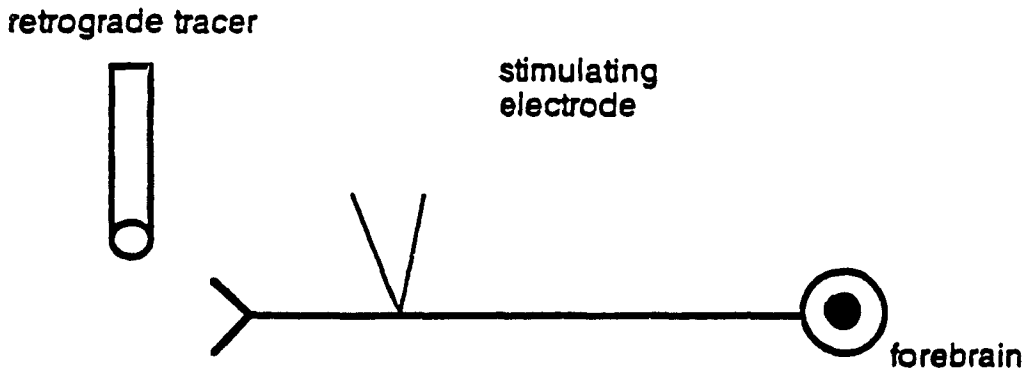
Several exogenous molecules are taken up by axonal terminals and are retrogradely transported towards the soma to eventually fill up the cytoplasm. Visualization of these 'retrograde tracers' by histochemical methods has provided invaluable information on neuronal pathways. However, the uptake of retrograde tracers is non-selective and their visualization does not provide any information about the chemical content of the neuron or its current metabolic state. In order to characterize the chemical identity or metabolic state of labeled cells, the use of retrograde tracers can be combined with neurochemical and/or metabolic markers. In the case of BSR studies, the combination of a retrograde tracer, administered in terminal regions of candidate cells, with Fos immunohistochemistry could reveal neurons that are antidromically activated. Neurons that antidromically activated by the MFB stimulating electrode *and* whose fibers terminate in the site in which the tracer was injected, will exhibit both Fos and the cytoplasmic retrograde tracer (Fig. 12a). Nonetheless, it is important to bear in mind that a double labeled candidate cell is not necessarily antidromically activated, but if its axon passes near the stimulating electrode, the likelihood of antidromic activation is high. Figure 12b shows a situation in which a transsynaptically-activated neuron would be double stained.

A pilot study has already tested the feasibility of the double labeling technique following LH self-stimulation. The retrograde tracer cholera toxin B subunit conjugated with colloidal gold (CTB-gold) was injected into the VTA which is a site where a large proportion of the anterior MFB fibers are known to terminate (Nieuwenhuys et al., 1982). Following one hour of LH self-stimulation the co-staining of Fos and the tracer was assessed. Several double labeled cells were found in anterior forebrain regions, corresponding to the ones that have already been proposed as candidate cells. As expected these were interspersed amongst cells that exhibited only Fos or CTB.

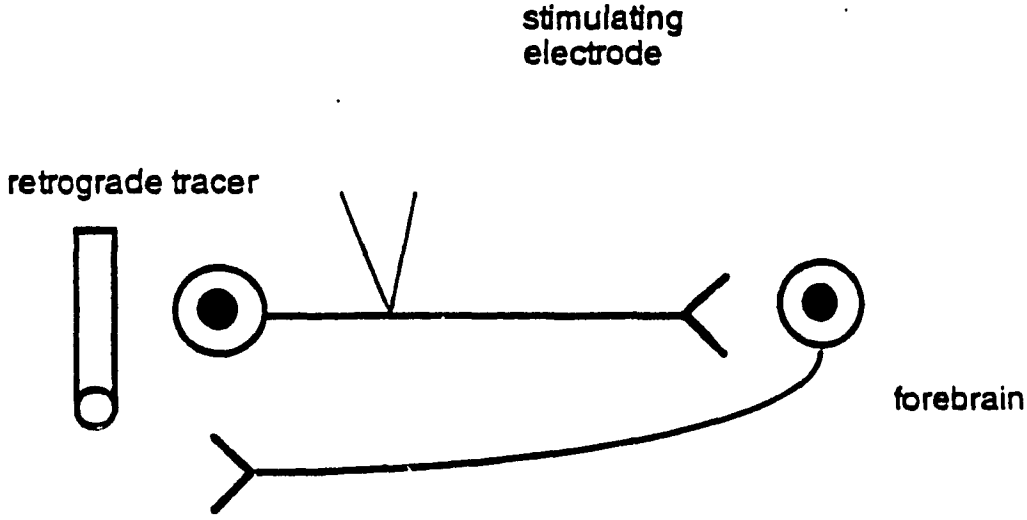
The use of CTB-gold as a retrograde tracer offers certain advantages for future studies. As already mentioned, the neurochemical identity of the first-stage neurons remains unknown. By combining Fos immunohistochemistry, retrograde tracing techniques, and immunohistochemical labeling for particular neuronal biochemical markers it may be possible to determine which neurotransmitters are involved in MFB self-stimulation. However, a key factor to the success of triple labeling is the compatibility of the three labeling methods.

Figure 12. **A.** Diagram of an antidromically activated neuron that will likely show both FLIR expression and labeling for the retrograde tracer. **B.** Diagram of a transsynaptically activated neuron that will likely exhibit both FLIR and retrograde tracer labeling.

A.


retrograde tracer
stimulating electrode
forebrain

B.


stimulating electrode
retrograde tracer
forebrain

Even though CTB-gold does not provide the best morphological resolution, its visualization as small black granules throughout the cytoplasm, not only permits the visualization of the dark stained nucleus, but of other immunohistochemical stains that uniformly label the cytoplasm. Future studies should be aimed at the visualization of triple labeled cells in order to obtain information about the neurochemical nature of the first-stage cells. This information will allow the implementation of pharmacological manipulations and of specifically targeted immunotoxins so as to conclusively determine the neurochemical identity of the first-stage.

References

Anderson, R., Fatigati, D., & Rompré, P. -P. (1995) Estimates of the axonal absolute refractory period of mesencephalic dopamine neurons. Society for Neuroscience Abstracts, 21, 176.

Arvanitogiannis, A., Flores, C., Pfaus, J. G., & Shizgal, P. Increased ipsilateral expression of Fos following lateral hypothalamic self-stimulation. Manuscript submitted for publication.

Arvanitogiannis, A., Kiscaldino, L., Shizgal, P. (1995). Effects of excitotoxic lesions of the medial basal forebrain on MFB self-stimulation. Society for Neuroscience Abstracts, 21, 176.

Arvanitogiannis, A., Waraczynski, M.,& Shizgal, P. Effects of excitotoxic lesions of the basal forebrain on MFB self-stimulation. Physiology and Behavior. In press.

Bielajew, C. (1991). Distribution of cytochrome oxidase in response to rewarding brain stimulation: effect of different pulse duration, Brain Research Bulletin, 26, 379-384.

Bielajew, C., & Shizgal, P. (1982). Behaviorally derived measures of conduction velocity in the substrate for rewarding medial forebrain bundle stimulation. Brain Research, 237, 107-119.

Bielajew, C., & Shizgal, P. (1986). Evidence implicating descending fibers in self-stimulation of the medial forebrain bundle. Journal of Neuroscience, 6, 919-929.

Blaha, C. D. & Phillips A. G. (1990). Application of in vivo electrochemistry to the measurement of change in dopamine release during intracranial self-stimulation. Journal of Neuroscience Methods, 34, 125-133.

Bozarth, M. A., & Wise, R. A. (1981). Intracranial self-administration of morphine into the ventral tegmental area of rats. Life Sciences, 28, 551-555.

Conover, K., & Shizgal, P. (1994a). Competition and summation between rewarding effects of sucrose and lateral hypothalamic stimulation in the rat. Behavioral Neuroscience, 108, 537-548.

Conover, K., & Shizgal, P. (1994b). Differential effects of postingestive feedback on the reward value of sucrose and lateral hypothalamic stimulation in rats. Behavioral Neuroscience, 108, 559-572.

Conover, K., Woodside, B., & Shizgal, P. (1994). Effects of sodium depletion on competition and summation between rewarding effects of salt and lateral hypothalamic stimulation in the rat. Behavioral Neuroscience, 108, 549-558.

De Olmos, J., Alheid, G. F. , & Beltramino, C.A. (1985). Amygdala. In: G. Paxinos (Ed.).The rat nervous system. Forebrain and midbrain. (Vol 1., pp. 223-334). Australia: Academic Press.

Domesick, V. B. (1981). Further observations of the nucleus accumbens and caudate putamen in the rat: similarities and contrasts. In: R. B. Chronister and J. F. De France (Eds.). The neurobiology of the nucleus accumbens, pp. 7-39. Haer Institute for Electrophysiologial Research.

Dragunow, M., Robertson, H.A. (1987). Kindling stimulation induces *c-fos* protein(s) in granule cells of the rat dentate gyrus. Nature, 329, 441-442.

Edmonds, D.E., & Gallistel, C.R. (1974). Parametric analysis of brain stimulation reward in the rat: III. Effect of performance variables on the reward summation function. Journal of Comparative and Physiological Psychology, 87, 876-883.

Fallon, J. H., & Moore, R. Y. (1978). Catecholamine innervation of the basal forebrain. Topography of the dopamine projection to the basal forebrain and neostriatum. Journal of Comparative Neurology, 180, 545-580.

Fibiger, H. C., LePiane, F. G., Jakubovic, A., & Phillips, A. G. (1987). The role of dopamine in intracranial self-stimulation of the ventral tegmental area. Journal of Neuroscience, 7, 3888-3897

Fouriezos, G., Wise, R. A. (1976). Pimozide-induced extinction of intracranial self-stimulation: response patterns rule out motor or performance deficits. Brain Research, 103, 377-380.

Gallistel, C.R., & Freyd, G. (1987). Quantitative determination of the effects of catecholaminergic agonists and antagonists on the rewarding efficacy of brain stimulation. Pharmacology, Biochemsitry, and Behaviour, 26, 731-741.

Gallistel, C. R., Gomita, Y., Yadin, E., & Campbell, K. A. (1985). Forebrain origins and terminations of the medial forebrain bundle metabolically activated by rewarding stimulation or by reward-blocking doses of pimozide. Journal of Neuroscience, 5, 1246-1261.

Gallistel, C. R.,& Karras, D. (1984). Pimozide and amphetamine have opposing effects on the reward summation function. Pharmacology Biochemistry & Behavior, 20, 73-77.

Gallistel, C. P., Shizgal, P., & Yeomans, J. S.(1981). A portrait of the substrate for self-stimulation, Psychological Reviews, 88, 228-273.

Glimcher, P. W., & Gallistel, C. R. (1989). Dorsomedialhypothalamic neurons give rise to most or all of the substrate for medial forebrain (MFB) self-stimulation. Society for Neuroscience, 15, 33.

Gogas, K. R., Levine, J. D., & Basbaum, A. I. (1993). Mechanisms of opioid-mediated antinociception: correlation of Fos expression and behavior. NIDA Research Monograph, 125, 25-38.

Gratton, A., & Wise, R. A. (1988a). Comparisons of refractory periods for medial forebrain bundle fibers subserving stimulation-induced feeding and brain stimulation reward: a psychophysical study. Brain Research, 438, 256-263.

Gratton, A., & Wise, R. A. (1988a). Comparisons of connectivity and conduction velocities for medial forebrain bundle fibers subserving stimulation-induce feeding and brain stimulation reward. Brain Research, 438, 264-270.

Heimer, L., Alheid, G. F., & Zaborszky, L. (1985). Basal Ganglia. In: G. Paxinos (Ed.).The rat nervous system. Forebrain and midbrain. (Vol 1., pp. 223-334). Australia: Academic Press.

Hoebel, B. G. (1969). Feeding and self-stimulation. Annals of the New York Academy of Sciences, 157, 758-778.

Hsu, S.M., Raine, L., & Fanger, H. (1981). The use of anti-avidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase techniques. American Journal of Clinical Pathology, 75, 816-821.

Janas, J.D., & Stellar, J.R. (1987). Effects of knife cut-lesions of the medial forebrain bundle in self-stimulation rats. Behavioural Neuroscience, 101, 832-845.

Jasmin, L. Gogas, K. R., Ahlgren, S.C., Levine, J. D., & Basbaum, A. I. (1994). Walking evokes a distinctive pattern of Fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat. Neuroscience, 58, 275-286

Johnson, P. I., & Stellar, J. R. (1994). NMDA-induced lesions of the nucleus accumbens and/or the ventral pallidum fail to attenuate lateral hypothalamic self-stimulation reward. Brain Research, 646, 73-84.

Kornhauser, J. M. , Dwight, E.N., Mayo, K. E., Takahashi, J. S., (1990). Photic and circadian regulation of *c-fos* gene expression in the hamster suprachiasmatic nucleus. Neuron, 5, 127-134.

Lindvall, O., & Björklund, A. (1974). The organization of the ascending catecholamine neurons systems in the rat brain as revealed by glyoxcyclic acid fluorescence method. Acta Physiologica Scandinavica (Suppl.), 412, 1-48.

Malette, J., & Miliaressis, E. (1994). Interhemispheric links in brain stimulation reward. Behavioural Brain Research, 68, 117-137.

Milliaressis, E., Rompré, P.-P., Laviolette, P., Phillippe, L. & Coulombe, D. (1986). The curve-shift paradigm in self-stimulation. Physiology and Behavior, 37, 85-91.

Morelli, M., Carboni, E., Cozzolino, A., Tanda, G. L., Pinna, A., & Di Chiara, G. (1992). Combined microdialysis and Fos immunohistochemistry for the estimation of dopamine neurotransmission in the rat caudate-putamen. Journal of Neurochemistry, 59, 1158-1160.

Morgan, J. I., & Curran, T. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. Annual Review of Neuroscience, 14, 421-51

Mundl, W. J.(1980). A constant-current stimulator. Physiology and Behavior, 24, 991-993.

Murray, B., & Shizgal, P. (1991). Anterolateral lesions of the medial forebrain bundle increases the frequency threshold for self-stimulation of the lateral hypothalamus and ventral tegmental area in the rat. Psychobiology, 19, 135-146.

Murray, B., & Shizgal, P. Behavioral measures of conduction velocity and refractory period for putative reward-relevant axons in the anterior LH and VTA. Physiology and Behavior. In press,a.

Murray, B. & Shizgal, P. Physiological measures of conduction velocity and refractory period for reward-relevant axons in the anterior LH and VTA. Physiology and Behavior. In press,b.

Murray, B. & Shizgal, P. Attenuation of medial forebrain bundle reward by anterior lateral hypothalamic lesions. Behavioral Brain Research. In press,c.

Nakahara, D., Fuchikami, K., Ozaki, N., Iwasaki, T., & Nagatsu, T. (1992). Differential effect of self-stimulation on dopamine release and metabolism in the rat medial frontal cortex, nucleus accumbens and striatum studied by in vivo microdialysis. Brain Research 574, 164-170.

Nieuwenhuys, R., Geeraeddts, L. M. G. , & Veening, J. G. (1982). The medial forebrain bundle of the rat. I. General introduction. Journal of Comparative Neurology, 206, 49-81.

Olds, J., & Milner, P. M. (1954). Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. Journal of Comparative and Physiological Psychology, 47, 419-427.

Paxinos, G., & Watson, C. The rat brain in stereotaxic coordinates (2nd ed.). Sydney: Academic Press.

Pfaus, J., Jakob, A., Kleopoulos, S. P., Gibb, R. B., & Pfaff, D. W. (1994). Sexual stimulation induces Fos immunoreactivity within GnRH neurons of the female rat preoptic area: Interaction with steroid hormones. Neuroendocrinology, 60, 283-290.

Phillipson, O. T. (1979). Afferent projections to the ventral tegmental area of Tsai and interfascicular nucleus: a horseradish peroxidase study in the rat. Journal of Comparative Neurology, 187, 117-143.

Porrino, L., Huston-Lyons, D., Bain, G., Sokoloff, L., & Kornetsky, C. (1990). The distribution of changes in local cerebral energy metabolism associated with brain stimulation reward to the medial forebrain bundle in the rat. Brain Research, 511, 1-6.

Rivier, C. L., Plotsky, P.M. (1986). Mediation by corticotropin releasing factor (CRF) of adenohypophysial hormone secretion. Annual Review of Physiology, 48, 475-494

Robertson, H.A. Immediate-early genes, neuronal plasticity, and memory. Biochemistry and Cell Biology. 70, 729-737.

Rompré, P.-P., & Shizgal, P. (1986). Electrophysiological characteristics of neurons in the forebrain regions implicated in self-stimulation of the medial forebrain bundle in the rat. Brain Research, 364, 338-349.

Rompré, P.-P., Wise, R. A. (1989). Opioid neuroleptic interaction in brain stem self-stimulation. Brain Research, 477, 144-151.

Routtenberg, A., & Lindy, J. (1965). Effects of the availability of rewarding septal and hypothalamic stimulation on bar pressing for food under conditions of deprivation. Journal of Comparative and Physiological Psychology, 60, 158-161.

Sagar, S.M., Sharp, F. R., & Curran, T. (1998). Expression of *c-fos* protein in the brain: metabolic mapping at the cellular level. Science, 244, 1328-1331.

Sharp, F. R., Hisanaga, K., & Sagar, S. M. (1993). NMDA receptor blockade prevents translation, but not transcription, of the *c-fos* gene following stimulation with multiple extracellular signals in cultured cortical neurons: implications for plasticity and molecular memory. NIDA Research Monograph, 125, 25-38.

Sheng, M., & Greenberg, M. E. (1990). The regulation and function of *c-fos* and other immediate early genes in the nervous system. Neuron, 4, 477-485.

Shizgal, P., Bielajew, C., Corbett, D., Skelton, R., & Yeomans, J. (1980). Behavioral methods for inferring anatomical linkage between rewarding brain stimulation sites. Journal of Comparative and Physiological Psychology, 94, 227-237.

Shizgal, P. & Murray, B. (1989). Neuronal basis of intracranial self-stimulation. In: J. M. Liebman and S.J. Cooper (Eds.), The neuropharmachological basis of reward. Oxford: Oxford University Press.

Shizgal, P. & Murray, B. (1994). The behavioural adaptation of the collision test: inference of axonal linkage and estimation of conduction velocity from effects of paired-pulse stimulation. Neuroscience Protocols, 94, 1-19.

Shizgal, P., Schindler, D., & Rompré, P.-P. (1989). Forebrain neurons driven by rewarding stimulation of the medial forebrain bundle in the rat: comparison of psychophysical and electrophysiological estimates of refractory periods. Brain Research, 499, 234-248.

Simerly, R. B., & Swanson, L. W. (1988). Projections of the medial preoptic nucleus: a *phaseolus vulgaris* leucoagglutinin anterograde tract-tracing study in the rat. Journal of Comparative Neurology, 270, 209-242..

Stellar, J. R., Hall, F.S., & Waraczynski, M. (1991). The effects of excitotoxin lesions of the lateral hypothalamus on self-stimulation reward. Brain Research, 541, 29-40.

Swanson, L. W. (1992). Brain maps: Structure of the rat brain, Elsevier, Amsterdam.

Swanson, L. W. (1992-4). Brain maps: Computer graphics files. Elsevier, Amsterdam.

Van Bockstaele, E.J., & Pickel, V. M. (1995). GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. Brain Research, 682, 215-221.

Waraczynski, M. (1988). Basal forebrain knife cuts and medial forebrain bundle self-stimulation. Brain Research, 438, 8-22.

Waraczynski, M.A., Ng Cheong-Ton, M., & Shizgal, P. (1990). Failure of amygdaloid lesions to increase the threshold for self-stimulation of the lateral hypothalamus and ventral tegmental area. Behavioral Brain Research, 40, 159-168.

Waraczynski, M.A., Conover, K., & Shizgal, P. (1992). Rewarding effectiveness of caudal MFB stimulation is unaltered following DMH lesions. Physiology and Behavior, 52, 211-218.

Wise, R. A. (1980). Actions of drugs of abuse on brain reward systems. Pharmacology Biochemistry and Behavior, 13, 213-223.

Wise, R. A. (1991). Neuroleptic-induced Anhedonia. Recent studies. In C. A. Tamminga and S. C. Schulz (Eds.), Advances in neuropshychiatry and psychopharmacology (Vol 1, pp. 323-331). New York: Raven Press, Ltd.

Wise, R. A., Bauco, P., Carlezon, W. A., Trojniar, W. (1992). Self-stimulation and drug reward mechanisms. Annals of the New York Academy of Sciences, 654, 192-198.

Wise, R. A., & Bozarth, M. A. (1982).Actions of drugs of abuse on brain reward systems: an update with specific attention to opiates. Pharmacology Biochemistry and Behavior, 17, 239-43.

Wise , R. A., & Colle, L. M. (1988). Effects of nucleus accumbens amphetamine on lateral hypothalamic brain stimulation reward. Brain Research, 459, 361-368.

Yeomans, J. S. (1979). The absolute refractory periods of self-stimulation neurons. Physiology and Behavior, 22, 911-919.

Yeomans, J. S., Mathur, A., & Tampakeras, M. (1993). Rewarding brain stimulation: role of tegmental cholinergic neurons that activate dopamine neurons. Behavioral Neuroscience, 107, 1-11.

Yeomans, J. S., Maidment, N. T., & Bunney, B. S. (1988). Excitability properties of medial forebrain bundle axons of A9 and A10 dopamine cells. Brain Research, 450, 86-93.

Yim, C., & Mogenson, G. (1980). Electrophysiological studies of neurons in the ventral tegmental area of Tsai. Brain Research, 181, 301-313.

Young, S.D., & Michael, A. C. (1993). Volatammetry of extracellular dopamine in rats striatum during ICSS-like electrical stimulation of the medial forebrain bundle. Brain Research, 600, 305-307.

Appendix I

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Stimulation site	1	60405.538	60405.538	8.680	.0146
error	10	69593.500	6959.350		
Area	14	538617.462	33472.676	31.928	.0000
S x A	14	72076.549	5148.325	4.273	.0000
error	140	168696.514	1204.975		
Hemisphere	1	326075.714	326075.714	247.648	.0000
S x H	1	6855.146	6855.146	5.206	.0457
error	10	13166.905	1316.691		
A x H	14	47502.655	3393.047	7.534	.0000
S x A x H	14	67948.678	4853.477	10.777	.0000
error	140	63048.771	450.348		

Appendix II

Source of variation	df	Mean Square	*F*	p
Hemisphere at VTA	1	119186.507	90.520	.000
error	10	1316.691		
Hemisphere at LH	1	213744.353	162.335	.000
error	10	1316.691		

Appendix III

Source of variation	df	Mean Square	F	p
Site of stimulation at OT	1	110.94	.056	.817
error	10	1972.237		
Site of stimulation at PFC	1	3914.260	1.487	.251
error	10	2633.201		
Site of stimulation at SEPT	1	18354.070	6.145	.033
error	10	2987.019		
Site of stimulation at NA	1	27948.375	13.831	.004
error	10	2020.725		
Site of stimulation at CP	1	2136.461	.566	.469
error	10	3771.915		
Site of stimulation at MPOA	1	5781.510	16.449	.002
error	10	351.474		
Site of stimulation at LPOA	1	17985.375	16.540	.002
error	10	1087.402		
Site of stimulation at BST	1	19814.507	28.565	.000
error	10	693.663		

Site of stimulation at LHA	1	3986.104	5.285	.044
error	10	754.215		
Site of stimulation at SI	1	1962.042	7.265	.022
error	10	270.084		
Site of stimulation at PVN	1	12082.594	7.682	.020
error	10	1572.751		
Site of stimulation at AMY	1	278.802	1.303	.280
error	10	213.996		
Site of stimulation at LHP	1	10803.527	11.742	.006
error	10	920.102		
Site of stimulation at DMH	1	7290.620	1.911	.197
error	10	3815.148		
Site of stimulation at VTA	1	32.9	.043	.840
error	10	765.068		

Appendix IV

OT

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	110.940	110.94	.056	.8173
error	10	19722.368	1972.237		
Hemisphere	1	5648.802	5648.802	33.673	.0002
S x H	1	807.360	807.360	4.813	.0530
error	10	167.755	167.755		

PFC

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	3914.260	3914.260	1.487	.250
error	10	26332.011	2633.201		
Hemisphere	1	9861.760	9861.760	20.620	.0011
S x H	1	5633.470	5633.470	11.779	.0064
error	10	4782.734	478.237		

SEPT

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	18354.070	18354.070	6.145	.0326
error	10	29870.194	2987.019		
Hemisphere	1	72018.170	72018.170	55.894	.0000
S x H	1	13071.334	13071.334	10.145	.0097
error	10	12884.691	1288.469		

NA

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	27948.375	27948.375	13.831	.004
error	10	20207.248	2020.725		
Hemisphere	1	16579.527	16579.527	64.329	.0000
S x H	1	.202	.202	.001	.9782
error	10	2577.302	257.730		

CP

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	2529.091	2529.091	.668	.4329
error	10	37884.648	3788.465		
Hemisphere	1	56489.896	56489.896	42.744	.0001
S x H	1	19200.424	19200.424	14.528	.0034
error	10	13215.848	1321.585		

MPOA

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	5781.510	5781.510	16.449	.0023
error	10	3514.744	351.474		
Hemisphere	1	6147.200	6147.200	65.094	.0000
S x H	1	1390.804	1390.804	14.728	.0033
error	10	944.351	94.435		

LPOA

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	17985.375	17985.375	16.540	.0023
error	10	10874.018	1087.402		
Hemisphere	1	23039.207	23039.207	61.55	.0000
S x H	1	10077.802	10077.802	26.923	.0004
error	10	3743.152	374.315		

BST

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	19814.507	19814.507	28.565	.0003
error	10	6936.632	693.663		
Hemisphere	1	19018.140	19018.140	59.866	.0000
S x H	1	6266.202	6266.202	19.725	.0013
error	10	3176.778	317.678		

LHA

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	3986.104	3986.104	5.285	.0443
error	10	7542.151	754.215		
Hemisphere	1	19385.850	19385.850	40.086	.0001
S x H	1	2511.260	2511.260	5.913	.0459
error	10	4866.044	483.604		

SI

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	1962.042	1962.042	7.265	.0225
error	10	2700.843	270.084		
Hemisphere	1	17259.207	17259.207	57.740	.0000
S x H	1	1380.167	1380.167	4.617	.0572
error	10	2989.127	298.913		

PVN

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	12082.594	12082.594	7.682	.0197
error	10	15727.508	1572.751		
Hemisphere	1	14215.534	14215.534	24.006	.0006
S x H	1	1409.134	1409.134	2.380	.1540
error	10	5921.768	592.177		

AMY

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	278.802	278.802	1.303	.2803
error	10	2139.962	213.996		
Hemisphere	1	6150.402	6150.402	56.778	.0000
S x H	1	250.907	250.907	2.316	.1590
error	10	1083.242	108.324		

LHP

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	10803.527	10803.527	11.742	.0065
error	10	9201.023	920.102		
Hemisphere	1	42993.735	42993.735	228.09	.0000
S x H	1	6383.082	6383.082	33.864	.0002
error	10	1884.913	188.491		

DMH

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	7290.620	7290.620	1.911	.1969
error	10	38151.481	3815.148		
Hemisphere	1	41110.204	41110.204	41.297	.0001
S x H	1	3382.000	3382.000	3.397	.0951
error	10	9954.711	995.471		

VTA

Source of variation	df	Sum of Squares	Mean Square	*F*	p
Site	1	32.900	32.900	.043	.8399
error	10	7650.684	765.068		
Hemisphere	1	25578.010	25578.010	38.125	.0001
S x H	1	1895.704	1895.704	2.826	.1237
error	10	6708.971	670.897		

Appendix V

PFC

Source of variation	df	Mean Square	*F*	p
Hemisphere at VTA	1	15201.201	31.783	.000
error	10	478.273		
Hemisphere at LH	1	294.030	.615	.451
error	10	478.273		

CP

Source of variation	df	Mean Square	*F*	p
Hemisphere at VTA	1	70778.88	53.556	.000
error	10	1321.585		
Hemisphere at LH	1	4911.439	3.716	.083
error	10	1321.585		

LPOA

Source of variation	df	Mean Square	*F*	p
Hemisphere at VTA	1	1320.901	3.529	.090
error	10	374.315		
Hemisphere at LH	1	31796.108	84.945	.000
error	10	374.315		