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The Role of the Locus Coeruleus and its Effect on Spontaneous Motor Activity in Response to Intraperitoneal Lipopolysaccharide in Rats

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The Role of the Locus Coeruleus and its Effect on Spontaneous Motor Activity in Response to Intraperitoneal Lipopolysaccharide in Rats

A Dissertation

Presented in Partial Fulfillment of Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University

2000

by

Katherine Ann Williams

ABSTRACT

The locus coeruleus (LC) is a group of norepinephrine (NE)-containing neurons that shows marked NE depletion when rats are exposed to uncontrollable shock. The depletion of NE in the LC region results in an increase in burst-firing of LC neurons. Evidence indicates that an increase in burst-firing of LC neurons results in a decrease in motor activity. Intraperitoneal (ip) administration of lipopolysaccharide (LPS), cell wall of gram-negative bacteria, also causes an increase in burst-firing of LC neurons, apparently via the induction of interleukin-1 (IL-1) in the brain. Ip LPS also results in a decrease in spontaneous motor activity in rats, a behavior that expressed in "sickness behavior." However, a causal relationship between an increase in burst-firing of LC neurons and a decrease in spontaneous motor activity after ip LPS has not yet been established. Therefore, this manuscript examines the effect of IL-1 acting within the LC region on producing an aspect of sickness behavior – a decrease in spontaneous motor activity -- that is seen after ip LPS. We hypothesize that the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region.

Several experiments conducted measured spontaneous motor activity as affected by 1) ip LPS, 2) LPS infused into the LC region or 3) IL-1 infused into the LC or LC surrounding regions. A final experiment tested whether interleukin-1 receptor antagonist (IL-1ra) infused into the LC region would block the decrease in spontaneous motor activity in response to an injection of ip LPS.

The results showed that a range of ip LPS doses (5-20 µg/kg), a range of LPS doses (0.1-4.0 ng/rat) or a range of IL-1β doses (0.1-40 ng/rat) infused into the LC region all decreased spontaneous motor activity in a dose-dependent manner. Unexpectedly, an infusion of IL-1β (0.4 ng/rat) into LC surrounding regions did not significantly decrease spontaneous motor activity differently from an infusion of IL-1β (0.4 ng/rat) into the LC region. Finally, central IL-1ra (40 µg/rat) was unable to block or affect the decrease in spontaneous motor activity seen after an injection of ip LPS (10 µg/kg).

Collectively, the findings do not support the hypothesis that the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region. Although LPS infused into the LC region or IL-1β infused into the LC region decreased spontaneous motor activity, IL-1ra was unable to block the decrease in spontaneous motor activity seen after an injection of ip LPS.

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LIST OF ABBREVIATIONS

4V – fourth ventricle
ANOVA – analysis of variance
AP – area postrema
AP (top of stereotaxic table (1) only) – Anterior/posterior
AQ – aqueduct
BBB – blood brain barrier
c – celsius
CaCl – calcium chloride
cm - centimeter
CSF – cerebral spinal fluid
dm-LC – dorsomedial to the locus coeruleus
DV - dorsoventral
Exp. - experiment
Fic-LPS – flourescein isothiocyanate LPS
GA – gauge
g – grams
hrs. – hours
ip – intraperitoneal(ly)
IL-1 – interleukin-1
IL-1α – interleukin-1 alpha
IL-1β – interleukin-1 beta
IL-1R - interleukin-1 receptor
IL-1ra - interleukin-1 receptor antagonist
irIL-1 – immunoreactive interleukin-1
KCl – potassium chloride
KDa – kilodalton
Kg – kilogram
LC – locus coeruleus
l-LC – lateral to the locus coeruleus
LPS – lipopolysaccharide
M - molar
MAOI - monoamine oxidase inhibitor
MgCl – magnesium chloride
MHPG – 3-methoxy-4-hydroxyphenylethylene glycol
min. - minute
ml - milliliter
m-LC – medial to the locus coeruleus
mm - millimeter
mM- millimolar
NaCl – sodium chloride
NE – norepinephrine
ng - nanogram

nl - nanoliter
NST – nucleus of the solitary tract
PE – polyethylene
RM-ANOVA – repeated measure analysis of variance
sec. - seconds
SEM – standard error of the mean
VEH - vehicle

CHAPTER ONE

Introduction

The introduction is divided into three parts. It begins by discussing the role of the locus coeruleus (LC) in mediating behavioral effects that result from exposure of rats to uncontrollable shock. Then, current evidence is presented showing that sickness behavior can be produced by lipopolysaccharide (LPS) or interleukin-1 (IL-1). Several related topics will discuss and provide supporting evidence for the proposed hypothesis. The final part of the introduction summarizes the project rationale, and briefly describes the experiments that were completed.

Part I

Part I of the introduction describes the locus coeruleus. This is followed by data showing that LC neuronal activity is regulated by noradrenergic (NE) input and that LC neuronal activity is increased in response to uncontrollable shock in rats. Finally, part I discusses how increased neuronal activity of the locus coeruleus affects motor activity in rats.

The Locus Coeruleus is a Noradrenergic Structure

The LC is a small nucleus of NE-containing neurons located in the pons ventral to the fourth ventricle (4V) and medial to the mesencephalic nucleus of the trigeminal cranial nerve. The axons of LC-NE neurons supply over 70% of all the NE in the brain (Dahlstrom et al., 1964; Huang et al., 1975), including almost all of the NE in the cerebral cortex and the hippocampus (Huang et al., 1975).

LC Neuronal Activity is Increased via "Functional Blockade" of α_2 Receptors in Response to Uncontrollable Shock

The LC appears to play an important role in mediating the behavioral effects that result from exposure of rats to uncontrollable shock (Simson et al., 1988; Weiss et al., 1996). Exposure to uncontrollable shock causes large depletions of NE in the LC region (Weiss et al., 1981). Under basal conditions, terminals from NE neurons release NE in the LC region and the NE activates adrenergic α_2 receptors that are located on NE cell bodies of the LC. The activation of α_2 receptors results in an inhibition of LC neuronal activity (Aghajanian et al., 1977; Cedarbaum et al., 1978a; Cedarbaum et al., 1978b). Conversely, non-activation of α_2 receptors results in a disinhibition of LC neuronal activity. Specifically, large depletions of NE in the LC region, produced by uncontrollable shock in rats, cause the release of NE in LC region to be decreased and α_2 receptors not to be normally activated by NE, i.e. the depletion of NE "functionally blocks" the α_2 receptors. The functional blockade of α_2 receptors results in a disinhibition of the LC, i.e. increased burst-firing (firing of several action potentials in rapid succession) of LC neurons. Several behavioral changes are caused by increased burst-firing of LC neurons, one of which is decreased motor activity (Simson et al., 1988).

LC Alpha$_2$ Receptors Affect Motor Activity in Rats

Alpha$_2$ Receptor Antagonists Decrease Motor Activity in Rats

Exposure to uncontrollable shock depletes NE in the LC region, functionally blocks α_2 receptors, and increase burst-firing of LC neurons, resulting in decreased motor

activity [measured by spontaneous motor activity (Weiss et al., 1980) or by a swim test (Simson et al., 1988)]. In nonstressed rats, an α_2 receptor antagonist (idazoxan) injected intravenously (Simson et al., 1987; Simson et al., 1989) or microinfused into the LC region (Simson et al., 1987) increased burst-firing of LC neurons, as seen after uncontrollable shock. Microinfusions of other α_2 receptor antagonists, such as piperoxane or yohimbine, into the LC region of nonstressed rats, also resulted in decreased motor activity (Weiss et al., 1986). These findings suggest that functionally blocking LC α_2 receptors by the depletion of NE, caused by uncontrollable shock or by administration of α_2 receptor antagonists, decreases motor activity in rats.

Activation of LC α_2 Receptors Blocks Decreased Motor Activity in Stressed Rats

Simson, Weiss, Ambrose, & Webster (1986a) found that decreased motor activity in rats exposed to uncontrollable shock could be blocked by an infusion of a monoamine oxidase inhibitor (MAOI) into the LC region. A MAOI inhibits the degradation of NE. Immediately following uncontrollable shock, rats were infused with pargyline (a MAOI) or a vehicle into the LC region. Motor activity, measured by a swim test, and NE concentrations in the LC region were measured two hours post-infusion of pargyline or vehicle. Stressed rats infused with a vehicle showed decreased motor activity and decreased NE concentrations in the LC region, when compared to unstressed rats. In contrast, stressed animals infused with pargyline (a MAOI) did not show a decrease in motor activity or a decrease in NE concentrations in the LC region. Pargyline was able to prevent the depletion of NE, normally seen after uncontrollable shock, resulting in motor activity scores similar to those of unstressed rats (Simson et al., 1986a).

Microinfusions of clonidine (α_2 receptor agonist) into the LC region also blocked the decreases in motor activity seen after uncontrollable shock (Simson et al., 1986b). Rats were exposed to either uncontrollable shock or no-shock and then returned to their home cages for 70 minutes. After the shock or no-shock session, the rats were infused with clonidine (α_2 receptor agonist) or a vehicle into the LC region. Immediately following the infusion, motor activity was measured using a swim test. Stressed rats infused with a vehicle showed decreased motor activity, when compared to nonstressed rats. In contrast, stressed rats infused with clonidine did not show decreased motor activity. The reactivation of α_2 receptors in the LC region with clonidine resulted in normal motor activity, similar to nonstressed rats infused with a vehicle (Simson et al., 1986b).

In summary, α_2 receptor antagonists infused into the LC region results in decreased motor activity of the rat. Conversely, α_2 receptor agonists into the LC region blocked the decrease in motor activity normally seen after exposure to uncontrollable shock. Collectively, these results suggest that decreases in motor activity are mediated via "functional blockade" of α_2 receptors located on NE neurons of the LC.

Part II

Part II of the introduction begins by discussing the behavioral similarities between the effects of human depression, exposure to uncontrollable shock in rats, bacterial infection in humans, and bacterial infection in rats. It is followed by a discussion of the physiological and behavioral effects that result from the administration of LPS, a specific bacterial derivative. The effects that are caused by LPS collectively can be termed

"sickness behavior," and these effects are mediated by IL-1. Evidence is presented showing that LPS causes the induction of IL-1. General information regarding the IL-1 system is presented prior to discussing how IL-1 also causes sickness behavior as seen after LPS. Finally, current evidence is presented showing that both LPS and IL-1 increase LC neuronal activity, as seen after exposure to uncontrollable shock.

Uncontrollable Shock, Bacterial Infections, and Sickness Behavior Result in Similar Behavioral Effects

Behavioral Effects of Depression, Uncontrollable Shock or Bacterial Infections

Human depression is delineated by some of the following symptoms: loss of appetite, change in sleep pattern, decrease in body weight (or a change in body weight), decrease in sex drive, decrease in energy levels, feelings of worthlessness, and recurrent thoughts of suicide (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, 1994, p. 320-327). Behaviors produced by exposure of rats to uncontrollable shock include: decreased social activities, decreased food and water intake, decreased body weight, and decreased motor activity (Weiss et al., 1982; Weiss et al., 1985). Bacterial infections also cause some of the symptoms seen after uncontrollable shock. Bacterial meningitis in humans has shown to cause weakness (Wispelwey et al., 1990). Peptidoglycan, a bacteria derived from *Neisseria gonorrhoeae*, causes significant decreases in food intake (Biberstine et al., 1996). It appears that depression in humans, exposure of rats to uncontrollable shock, and bacterial challenges all are characterized by similar behavioral changes.

Effects of LPS: Sickness Behavior

LPS, the outer lipid membrane of gram-negative bacteria derived from *Escherichia coli*, is a large molecule that does not cross the blood brain barrier (BBB) (Schmahl et al., 1974). LPS injected ip causes several behavioral changes that collectively can be termed "sickness behavior". Sickness behavior can be defined as a collection of physiological and behavioral changes that occur in response to an infectious substance being introduced into the body. Some of the physiological changes caused by LPS include: fever (Kozak et al., 1994; Kozak et al., 1995; Luheshi et al., 1996; Miller et al., 1997), increased plasma corticosterone (Dunn., 1992)(Jones et al., 1958)(Melby et al., 1960)(Wexler et al., 1957) as cited in (Dunn., 1992), and increased central interleukin-1 (IL-1) (Maier et al., 1993; Quan et al., 1994; Quan et al., 1997; Van Dam et al., 1995). Specifically, LPS causes a large increase in IL-1β mRNA (Ilyin et al., 1998) for prolonged periods followed by the direct translation into bioactive IL-1β (Dinarello., 1994). Among the behavioral changes caused by LPS are anhedonia [characterized by decreased preference for a saccharin solution in fluid-deprived rats (Yirmiya., 1996)], increased pain sensitivity [hyperalgesia (Maier et al., 1993; Walker et al., 1996a; Walker et al., 1996b; Watkins et al., 1994a; Watkins et al., 1994b; Watkins et al., 1994c)], decreased food intake (Aubert et al., 1997; Bret-Dibat et al., 1997a; Bret-Dibat et al., 1997b; Ilyin et al., 1998; Kent et al., 1996), and decreased social exploration (Bluthe et al., 1992; Bluthe et al., 1994). LPS given ip also decreases spontaneous motor activity in mice (Kozak et al., 1994; Kozak et al., 1995; Laye et al., 1995) and in rats (West, C. K. &

Weiss, J. M., unpublished results). The primary dependent measure and the main focus of interest throughout this manuscript is dark period spontaneous motor activity in rats.

<u>Central and Peripheral Induction of IL-1 in Response to LPS</u>

Infection or inflammation causes an immune response. Among the immune responses to an endotoxin such as LPS is the expression of *in vitro* and *in vivo* IL-1 in the periphery and in the brain. *In vitro* studies have shown that LPS stimulates the production of IL-1 (Fontana et al., 1982; Loughlin et al., 1996) and IL-1 mRNA in macrophages (Hetier et al., 1988; Loughlin et al., 1996). LPS also stimulates the production of IL-1 and IL-1 mRNA in cultured microglia (Fontana et al., 1982; Guilian et al., 1986; Hetier et al., 1988; Loughlin et al., 1996).

In vivo studies show that ip LPS increases IL-1β plasma levels and increases the expression of IL-1β mRNA in mouse brain (Laye et al., 1995). LPS increased immunoreactive IL-1 (irIL-1) produced by microglial cells and IL-1 mRNA in rat brain as soon as 30 minutes post-injection. The induction of irIL-1 and IL-1 mRNA, in response to LPS, was found in the meninges, choroid plexus, and circumventricular organs (Buttini et al., 1995; Quan et al., 1997; Van Dam et al., 1995; Van Dam et al., 1992). The induction of brain IL-1 in response to LPS occurred whether LPS was administered centrally or peripherally (Quan et al., 1994; Weiss et al., 1994).

<u>LPS-Sickness Behavior may be Caused by IL-1</u>

Several researchers suggest that IL-1 mediates the physiological and behavioral effects of LPS. This is supported by the following results: 1) LPS causes the expression of IL-1 in the brain (Quan et al., 1994; Weiss et al., 1994), 2) ip LPS causes a greater

febrile response in normal mice than in IL-1β-deficient mice (Kozak et al., 1995), 3) an interleukin-1 receptor antagonist (IL-1ra) decreased the magnitude and delayed the onset of fever produced by ip LPS (Luheshi et al., 1996) or by LPS given subcutaneously (Miller et al., 1997), 4) IL-1ra blocked the hyperalgesic response normally seen after LPS (Maier et al., 1993), and 5) administration of IL-1ra blocked the decrease in social exploration normally seen after ip LPS (Bluthe et al., 1992).

<u>Interleukin-1, Interleukin-1 Receptor Antagonist, and Interleukin-1 Receptors</u>

IL-1 is a cytokine that responds to infections or inflammations, and it is found both in the periphery and in the brain. IL-1 is a large 17-kDa molecule that may (Banks et al., 1989) or may not cross the blood brain barrier (Coceani et al., 1988). The IL-1 system contains three major components: IL-1, IL-1 receptor antagonist (IL-1ra), and IL-1 receptors (IL-1R) (Dinarello., 1994). IL-1 is expressed in two forms: IL-1α and IL-1β, both of which are synthesized as precursor proteins that result in IL-1α and IL-1β when the precursors are cleaved. The IL-1α precursor and the cleaved form of IL-1α both are biologically active; however, the IL-1β precursor is not biologically active. IL-1ra also is synthesized as a precursor protein and must be cleaved prior to being secreted as biologically active. IL-1ra is an endogenous cytokine and competes with IL-1 at the level of the IL-1R; however, binding of IL-1ra to the IL-1R will not initiate a biological response (Dinarello., 1994; Dripps et al., 1991). There are two types of IL-1 receptors: IL-1R type I and IL-1R type II. IL-1R type I contains a single transmembrane domain and will transduce a signal when activated by IL-1α or IL-1β. There are less than 100 IL-1R type I on most cells; however, only 2-3% of the IL-1R type I need to be occupied

for a biological response to occur. Although IL-1R type II also contains a single transmembrane domain, it will bind IL-1 but the receptor will not transduce a signal. In general, IL-1α and IL-1ra have a greater affinity for the IL-1R type I and IL-1β has a greater affinity for the IL-1R type II; however, all three forms of IL-1, (IL-1α, IL-1β or IL-1ra), will bind to both receptor types (Dinarello., 1994; Dripps et al., 1991).

Bioactive IL-1 is normally present in the cortex, diencephalon, hippocampus and brain stem (Quan et al., 1996). IL-1β mRNA has been detected in the cortex, hippocampus, hypothalamus, cerebellum (Ilyin et al., 1998; Ilyin et al., 1996), and brain stem (Taishi et al., 1997). IL-1R type I mRNA is found in the hypothalamus, hippocampus, cortex, cerebellum, and brain stem of rats (Gayle et al., 1997b). Several other researchers also have found IL-1R type I mRNA throughout the brain and choroid plexus of normal rats (Ericsson et al., 1995; Farrar et al., 1987; Ilyin et al., 1998; Ilyin et al., 1996; Wong et al., 1994; Yabuuchi et al., 1994). Although IL-1R type I mRNA is expressed mostly in non-neuronal cells, some researchers have detected IL-1R type I mRNA in neuron-like cells in the hypothalamus, trigeminal nucleus (Ericsson et al., 1995), thalamic nucleus (Yabuuchi et al., 1994), hippocampus, and cerebellar cortex (Wong et al., 1994). It should be noted that rat brain IL-1Rs have only been detected at the level of mRNA, since there are no commercially available rat IL-1R antibodies. Although it is likely that the detection of IL-1R mRNA is correlated with functional IL-1R, the actual relationship between IL-1R type I mRNA and functional IL-1R remains to be determined.

IL-1 and its Role in Sickness Behavior

IL-1 causes sickness behavior and induces physiological and behavioral effects that are similar to the behavior seen after the administration of LPS (see above). Some of the physiological effects caused by IL-1 include: fever (Kent et al., 1992; Watkins et al., 1995), long term peripheral immune responses (Quan et al., 1994; Sundar et al., 1989; Weiss et al., 1994), increased plasma corticosterone (Dunn., 1988; Dunn., 1992; Weiss et al., 1994), and large increases in central IL-1β mRNA (Ilyin et al., 1996). Among the behavioral changes caused by IL-1 are: hyperalgesia (Oka et al., 1993; Watkins et al., 1994c), decreased social exploration (Kent et al., 1992), and decreased food intake (Gayle et al., 1997a; Kent et al., 1992; Kent et al., 1996; Plata-Salaman et al., 1992). In addition, IL-1β or IL-1β-163-171-fragment infused into the LC caused behavioral sedation as characterized by electrocortical recordings (De Sarro et al., 1997) or by spontaneous motor activity (Montkowski, et al., 1997).

IL-1 Sickness Behavior can be Attenuated by IL-1ra

Several researchers suggest that the physiological and behavioral effects seen after IL-1 and/or LPS are mediated by IL-1 and the IL-1R. This is supported by the following results: 1) IL-1ra blocked or attenuated behavioral effects of ip LPS (see above). 2) IL-1ra blocked the hyperalgesic response normally seen after ventricular infusions of IL-1 (Oka et al., 1993), 3) IL-1ra attenuated the fever produced by IL-1 (Kent et al., 1992; Watkins et al., 1995), 4) IL-1ra blocked the decreases in social exploration normally seen after ip IL-1 (Kent et al., 1992), and 5) central IL-1ra blocked

the decreases in food intake normally seen after ventricular IL-1 (Kent et al., 1992; Kent et al., 1996; Plata-Salaman et al., 1992).

<u>LPS or IL-1 and their Effects on Neuronal Activity</u>

LPS causes *c-fos* expression, a marker of neuronal activity, in several brain stem areas including: the area postrema (AP), the nucleus of the solitary tract (NST), and the LC (Hare et al., 1995; Sagar et al., 1995; Wan et al., 1993). Similarly, LPS and IL-1 alter the metabolism of central NE as seen by a marked increase in the content of 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) (Dunn., 1988; Dunn., 1992; Kabiersch et al., 1988; Linhorst et al., 1998). An increase in MHPG, a metabolite of NE suggests an increase in NE neuronal activity.

LPS and IL-1 affect LC neuronal activity. IL-1 microinfused into the LC region increased burst-firing of LC neurons (Borsody., 1996) as seen after uncontrollable shock in rats. Ip LPS or microinfusions of LPS into the LC region also causes LC neurons to increase their burst-firing (Borsody., 1996), similar to the effects seen after IL-1 infusions into the LC region or the administration of uncontrollable shock in rats. The increase in burst-firing of LC neurons after ip LPS appears to be due to the induction of brain IL-1 in the LC region, initiated by the LPS. This is supported by the finding that IL-1ra microinfused into the LC region blocks the increase in burst-firing of LC neurons normally seen after an injection of ip LPS (Borsody., 1996).

The increase in LC neuronal activity seen after LPS or IL-1 is similar to the LC response after uncontrollable stress in rats. The increase in burst-firing of LC neurons, produced by uncontrollable shock in rats, is thought to mediate the decrease in motor

activity as measured by a swim test. Therefore, it is possible that the increase in burst-firing of LC neurons produced by ip LPS is responsible for the decrease in spontaneous motor activity seen after ip LPS.

<center>Part III</center>

Part III summarizes the introduction by discussing the role of the LC in response to uncontrollable shock, LPS, and IL-1. Furthermore, it states the project hypothesis and briefly describes the experiments that were completed.

Project Rationale

The LC is a group of NE-containing neurons whose axons supply over 70% of all the NE in the brain. The LC region shows marked depletion of NE when rats are exposed to uncontrollable shock. The depletion of NE results in a disinhibition of NE neurons of the LC and this disinhibition is expressed as an increase in burst-firing of LC neurons. The increase in burst-firing of LC neurons results in a decrease of motor activity in rats. Ip LPS causes a similar increase in burst-firing of LC neurons and a decrease in spontaneous motor activity. The increase in burst-firing of LC neurons seen after ip LPS is likely due to the induction of IL-1 in the LC region initiated by the LPS. This is supported by the following: 1) ip LPS causes the induction of IL-1 in the brain, 2) IL-1 microinfused into the LC region increases burst-firing of LC neurons, and 3) IL-1ra microinfused into the LC region blocks the increase in burst-firing of LC neurons normally seen after ip LPS. However, a causal relationship between the increase in burst-firing of LC neurons after ip LPS and the decrease in spontaneous motor activity produced by ip LPS has not yet been established. Therefore, this manuscript examines

the effect of IL-1 acting within the LC region on producing a decrease in spontaneous motor activity after ip LPS. **We hypothesize that the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region.**

Preliminary results collected in our laboratory showed that ip LPS decreases spontaneous motor activity in rats. Exp. 1 tests whether a range of ip LPS doses would cause a dose-related decrease in spontaneous motor activity. Exp. 2 tests whether a range of LPS doses infused into the LC region would decrease spontaneous motor activity in a dose-dependent manner. LPS is administered into the LC region because previous results indicate that ip LPS causes the induction of IL-1 in microglia and that physiological and behavioral effects of LPS are mediated by IL-1 in the brain. Exp. 3 tests whether a range of IL-1 doses microinfused into the LC region would decrease spontaneous motor activity as seen after ip LPS and whether the effects of IL-1 are dose-dependent. Exp. 4 tests whether the predicted decrease in spontaneous motor activity after an infusion of IL-1 into the LC region would be specific to the LC. This was done by assessing whether the decrease in motor activity would occur only if IL-1 were infused into the LC region and would not occur if IL-1 were infused into regions surrounding the LC. Exp. 5 measures changes in food and water intake, home-cage behaviors (digging, grooming, sitting, etc.), swim-test behaviors (struggling and floating) in addition to spontaneous motor activity in response to IL-1 infused into the LC region. Exp. 6 tests whether IL-1ra infused into the LC region would block the expected decrease in spontaneous motor activity after ip LPS.

CHAPTER TWO

General Methods

<u>Procedures</u>

<u>Animals</u>

Female (Exp. 1) and male (all other experiments) pathogen-free Sprague-Dawley rats (derived from Charles River Laboratories) were selected from our group-housed colony. Three to four month female rats weighed 200-350 grams (g) at the time of testing; and two to six month old male rats weighed 200-650 g at the time of testing. All animals were single housed in a room dedicated to monitoring of spontaneous motor activity by a computer-controlled system. Animals were placed into single housing for 3-4 days prior to measuring dependent variables or the surgical procedure. The room temperature was maintained at ~22°C with a 12:12 light: dark period (lights on from 0700-1900 hours (hrs.), unless noted otherwise. Purina rodent chow (No. 5001) and tap water were provided *ad libitum*. Deviations from the above procedure are detailed in their respective experimental methods section

<u>Surgical Procedures</u>

Animals were anesthetized with Halothane (3% in 100% oxygen, 3 liters/minute flow rate), the fur at the top of the head was shaved, and they were placed in the stereotaxic apparatus (David Kopf Instruments). A skin incision was made, a hole drilled in the skull, and dura mater was exposed in order to implant the cannula. The stereotaxic coordinates used to implant the cannulae are given in Table 1 and shown in Figure 1. The brain regions of interest are the (AQ), the locus coeruleus (LC), medial to the

Stereotaxic Coordinates

Structure	Nose angle	AP (from Lambda)	AP (from Bregma	ML	DV
AQ	-3.3^O	0 mm	-8.30 mm	0 mm	Approx. -5.0
LC	-10.0^O	-3.6 mm	-9.30 mm	+/- 1.1 mm	-7.0
m-LC	-10.0^O	-3.6 mm	-9.30 mm	0 mm	-7.2
l-LC	-10.0^O	-3.6 mm	-9.30 mm	+/- 2.0 mm	-6.7
4V	-3.3^O	-2.7 mm	-11.60 mm	0 mm	15.0^O tilt at –8.0
AP/NST	-3.3^O	-4.0 mm	-13.68 mm	0 mm	15.0^O tilt at –8.0

Table 1. Stereotaxic coordinates used to implant a cannula (Paxinos & Watson, 1997). Coordinates for the aqueduct (AQ), the locus coeruleus (LC), medial to the LC (m-LC), lateral to the LC (l-LC), the fourth ventricle (4V), and the area postrema/nucleus of the solitary tract region (AP/NST).
Anterior/posterior (AP) coordinates actually used are those referenced from the Lambda intersection. Equivalent coordinates from Bregma are provided as reference points in the stereotaxic atlas. Medial/lateral (ML) coordinates are referenced from the midline suture. The dorsal/ventral (DV) coordinates are referenced from dura mater to the implanted tip of the beveled cannula needle. The actual DV coordinate for the AQ was determined by the presence of cerebral spinal fluid (CSF) in the cannula. The DV coordinate for the 4V and the AP/NST are with the universal stereotaxic arm set at a 15° forward tilt.

AQ, Bregma –8.30 mm

LC, Bregma –9.30 mm

m-LC, Bregma –9.30 mm

l-LC, Bregma –9.30 mm

4V, Bregma –11.60 mm

AP/NTS, Bregma –13.68 mm
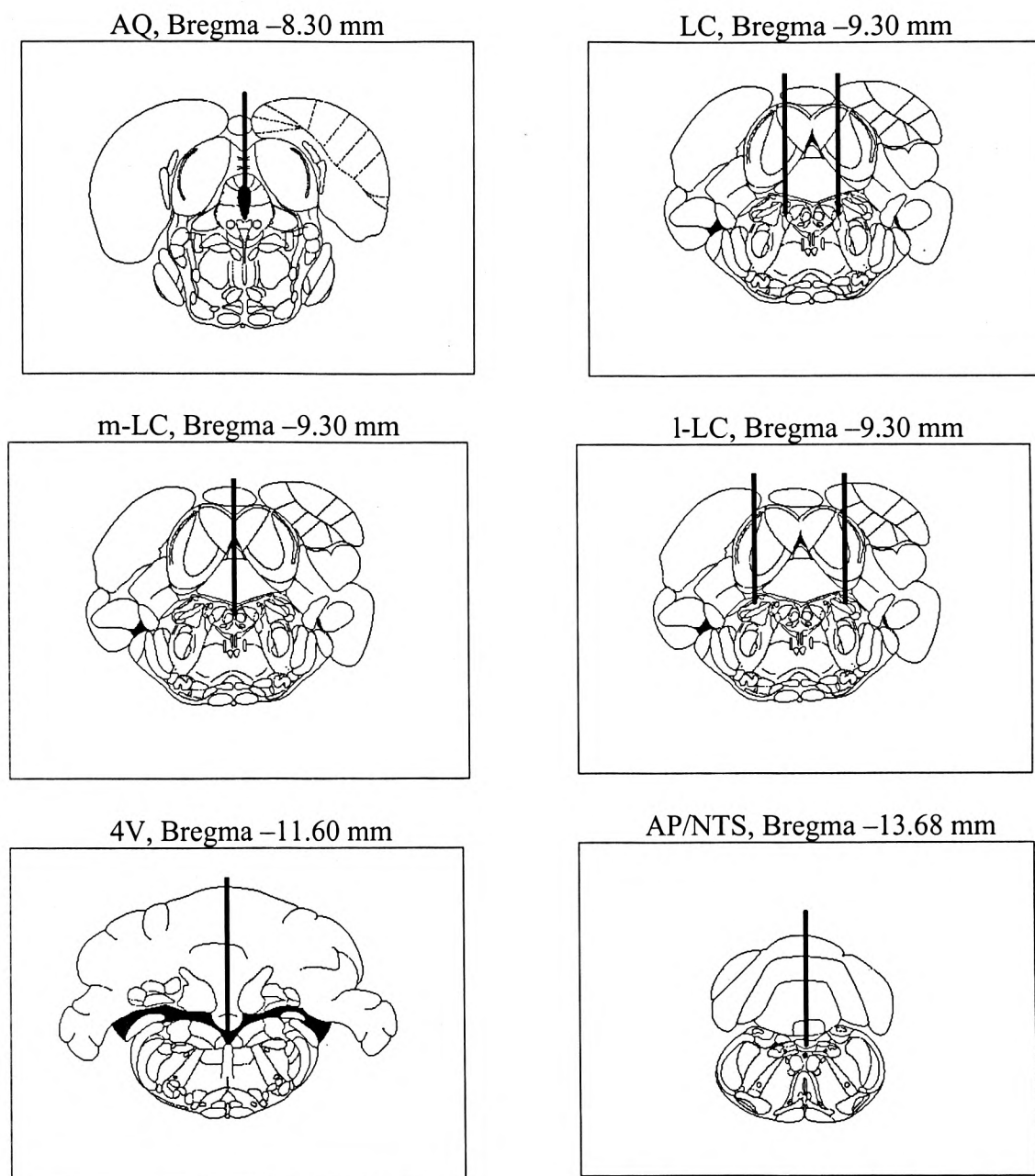
Figure 1. Representative coronal view of cannulae in the AQ, LC, m-LC, l-LC, 4V or the AP/NST.

[1]From The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1997, New York: Academic Press. Copyright © 1997 by Academic Press. Adapted with permission by Academic Press.

LC (m-LC), lateral to the LC (l-LC), the fourth ventricle (4V), and the area postrema/nucleus of the solitary tract (AP/NST). Each cannula was secured to the skull using 3/64 inches jeweler's screws and dental acrylic. Suture silk was used to close the skin incision made during surgery. The animals were returned to their home cage and given a 10-12 day recovery period prior to the administration of a drug.

Handling Procedures

Prior to any experimental procedure, animals were handled for 1-2 min./day, between 1700-1900 hrs. for a total of three days. Animals that were to receive an ip injection were handled so that their abdomen was exposed, as would occur when administering an ip drug. Animals that were to be infused were handled by gently restraining them, as it would occur during the infusion procedure. Following the handling process, spontaneous motor activity was recorded during the dark period and a three-day mean of motor activity was calculated. The three-day mean of motor activity is referred to as "baseline" motor activity. Deviations from the above procedure are detailed in their respective experimental methods section. Baseline motor activity was used to divide the animals into matched groups.

Spontaneous Motor Activity

In anticipation of the year 2000 and the potential computer/software problems that might have occurred, the computer software program that monitored spontaneous motor activity was updated after the completion of Exps. 1, 2-Part I, 3, 4 and 6. Experiments 2-Part II and Exp. 5 were conducted with an updated version of the computer software that measured spontaneous motor activity.

General Procedures for all Experiments

Animals were single housed and spontaneous motor activity was monitored by a computer-controlled system. The cages used while recording motor activity are standard 45 X 25 X 20 cm polycarbonate cages with wire lids. The cages contained ¼-inch-corncob bedding that was in direct contact with the animal. Spontaneous motor activity was recorded by eight infrared beams, spaced 5 cm apart, positioned in a horizontal frame that surrounds the home cage of the animal (ANA 1219, Riverpoint Electronics). Spontaneous motor activity represents general horizontal motor activity of the animal and is expressed as ambulatory counts (defined below) per hour. Repetitive movements by the animal in the same location are not counted as spontaneous motor activity. Spontaneous motor activity during the dark period is the primary dependent variable measured throughout the experiments in this manuscript.

Experiments 1, 2-Part I, 3, 4 and 6

Spontaneous motor activity (ambulatory counts) was monitored by two software programs; a DOS version of Crosstalk Script (programmed by Bob Bonsall) and a BASIC program that connected each cage to the computer-controlled system that calculated ambulation. A change in any of the eight infrared beams (being broken or unbroken) was recorded as a "sentence" by the computer. Each sentence represented the state of all eight infrared beams. Infrared beam changes were calculated by the computer and expressed as ambulatory counts (spontaneous motor activity). An ambulatory count was defined as a change in an infrared beam that had remained unchanged in the previous four "sentences".

Experiment 2-Part II and Experiment 5

The new computer-controlled system only required one software package to record motor activity (Labview Version 5.1 programmed by Bob Bonsall of Circular Solutions using Windows 98 as an operating system). A change in any of the eight infrared beams (being broken or unbroken) was recorded as a "sentence" by the new computer software. Each sentence represented the state of all eight infrared beams. Infrared beam changes were calculated by the computer and expressed as ambulatory counts. The new computer software defined an ambulatory count as a change in an infrared beam that had remained unchanged in the previous five "sentences".

Cannula and Infusion Needle Construction

Representations of an implanted cannula, an infusion needle/tubing, an attached microsyringe and microsyringe driver are shown in Figure 2; see Appendix A for detailed information. Each cannula was constructed from a 26 gauge (GA) intradermal bevel needle. The non-beveled end of the needle was attached to a narrow piece of silicone tubing, 5 cm in length. Also attached to the non-beveled end of the needle and covering the narrow tubing was a larger piece of silicone tubing, exactly 3.2 cm in length. To prevent clogging of the cannula after it was implanted, an obturator was inserted into the narrow tubing and held in place with silicone glue.

Drug delivery occurred via a stainless steel infusion needle attached to a microsyringe driver with polyethylene (PE) tubing. The drug was loaded into the tip of the infusion needle and the infusion needle was then inserted into the exposed portion of
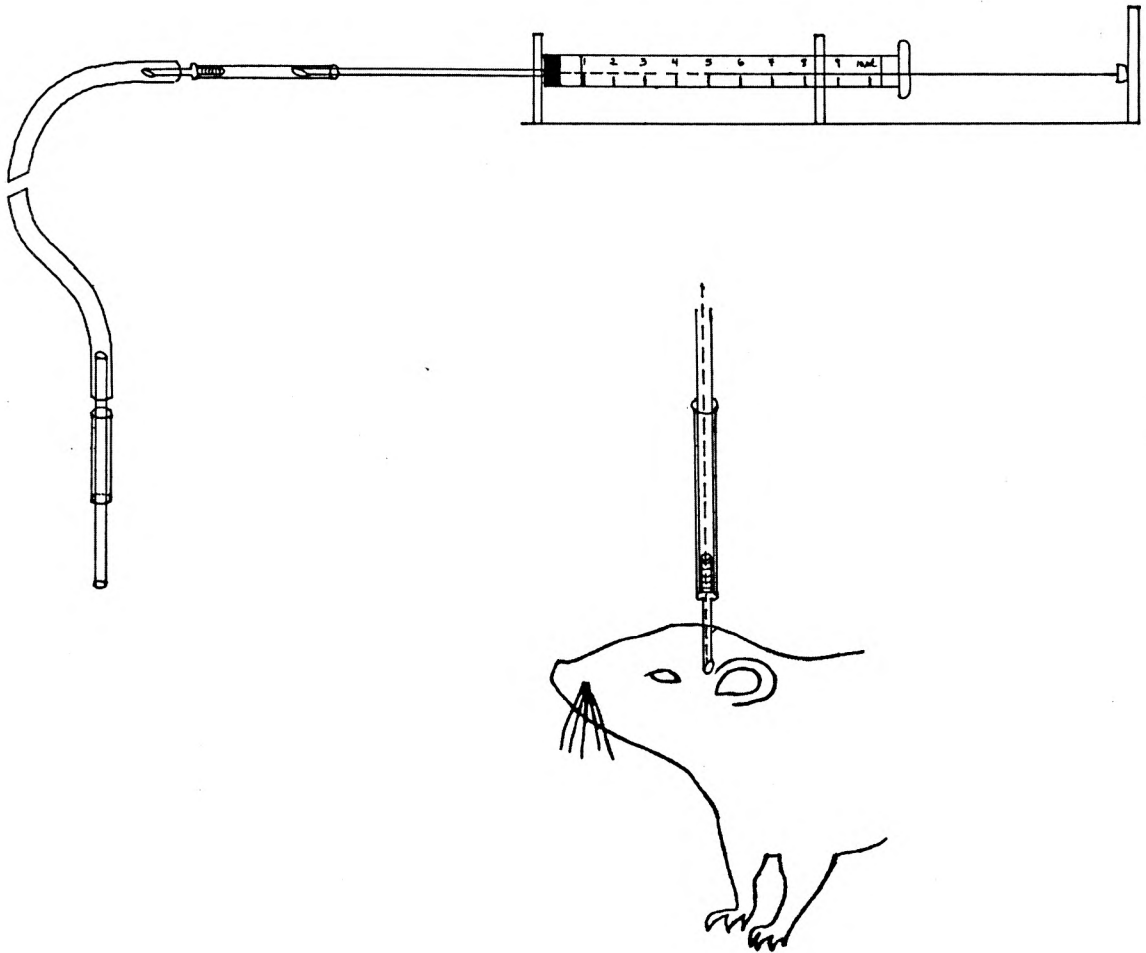
Figure 2. Representations of an implanted cannula (bottom right), an infusion needle/tubing (left) and an attached microsyringe driver (top right).

narrow silicone tube that was part of the implanted cannula. The microsyringe driver that holds two 10µl Hamilton syringes, was used to measure the amount of drug infused.

Infusion Procedure

The infusion procedure began prior to lights off and occurred adjacent to the room where motor activity was measured. Specific times of infusion are detailed in the respective experimental methods section. One animal at a time was transported in their home cage to the infusion room and gently restrained. The narrow tube of each cannula was cut flush with the end of the large silicone tube and the infusion needle was then inserted into the narrow silicone tube of the implanted cannula; see Figure 2 and Appendix A. The animal was returned to its home cage and remained there for the duration of the infusion (~5 min.). During the first minute of the infusion, the drug is advanced through a 2 µl "deadspace". The deadspace is measured in volume and is defined as the space between the end of the inserted infusion needle and the tip of the implanted cannula resting in the brain. Once the drug has passed the deadspace and when infusing a midline structure, 2 µl were infused at a rate of 500 nl/min. When infusing a bilateral structure, 1 µl/side was infused at a rate of 250 nl/min. The infusion needle was left in place for 30 seconds to avoid a vacuum effect upon removal. The animal was removed from its home cage, the infusion needle was removed and the end of the exposed silicone tubing was sealed with an electrical pin connector. The animal was again returned to its home cage and the cage was transported back into the room where motor activity was measured for 12 hrs. post-infusion.

Verification of Cannula Placement

Animals were anesthetized with Halothane, decapitated, and their brains were removed. A few brains were quickly dissected into 1 mm sections using a coronal brain blocker and cannulae placements were noted in a written form or recorded on section reproductions from the stereotaxic atlas. Unless noted otherwise, the majority of the brains were post-fixed in 4% paraformaldehyde until they were cut at 120 μm on a freezing microtome. The sections were mounted on slides, viewed under a microscope and cannulae placements were recorded on section reproductions from the stereotaxic atlas. Cannulae placements that were considered "misses" were omitted from statistical analyses and data presentation, unless otherwise noted.

Drugs

Intraperitoneal procedures

Dulbecco's phosphate buffered salt solution 1X (VEH) was used as a vehicle in a volume of 1.0 ml/kg of body weight. LPS (Sigma, L-3129) was diluted with VEH on the day injections were given. Specific LPS doses are detailed in respective experimental methods section.

Infusion procedures

Artificial cerebral spinal fluid (CSF) that contained 0.128M NaCl, 1.7 mM CaCl, 2.68 mM KCl and 0.98 mM MgCl was used as a vehicle for infusions. One to two days prior to the infusion procedure, recombinant rat interleukin-1β [(IL-1β), R & D Systems], interleukin-1 receptor antagonist [(IL-1ra) a gift from Synergen, Inc.], LPS (Sigma, L-3129) and Fluorescein Isothiocyanate LPS [(fic-LPS), Sigma, F-3540] were diluted in

CSF and stored at 4°C until used. Specific doses are detailed in respective experimental methods section.

Experimental Replications

Due to the number of animals in each study, it was necessary to conduct most experiments as a set of replications. Each replication used the same criteria and methodological procedures as the other replications within an experiment. In a single experiment, each replication contains a representative sample of the different groups. After appropriate statistical analyses, the data from each replication were combined and treated as a single data set, unless otherwise noted.

General Statistical Analyses

Experiments 1, 2-Part I, 3, 4 and 6

Pre-baseline (Exp. 1) or baseline (Exps. 2-4, 6) motor activity for each experiment was analyzed by a repeated measures analysis of variance (RM-ANOVA). These analyses were conducted to insure that pre-baseline (Exp. 1) or baseline (Exps. 2-4, 6) motor activity of the different groups were not statistically different. Post-injection (Exp. 1), post-infusion (Exps. 2-4) or post-infusion/injection (Exp. 6) motor activity were analyzed by a RM-ANOVA followed by separate two-way ANOVAs (Exp. 1) or one-way ANOVAs (Exp. 2-4, 6) at each hour of the dark period. *A priori* analyses were calculated with SPSS Statistical Software V 8.2. Following the ANOVA and where appropriate, *post hoc* analyses were conducted by a Dunnett test. Data are considered to reach statistical significance if $p < 0.05$, unless otherwise stated and reported in

respective results sections. Specific statistical significance's are reported in the respective result section each experiment.

Experiment 2-Part II and Experiment 5

Exp. 2-Part II does not require statistical analyses. Descriptions of the histological data are presented in its respective results section. Statistical analyses for Exp. 5 were calculated with SPSS Statistical Software V 8.2 (RM-ANOVAs) or Sigma Stat for Windows V 2.0 (Two-way ANOVAs) and are detailed in its respective methods section.

CHAPTER THREE

Experiment 1: Intraperitoneal LPS

Rationale

Preliminary results, collected in our laboratory, have shown that ip LPS (10 μg/kg) decreases spontaneous motor activity. Exp. 1 tested whether a range of ip LPS doses would cause dose-dependent decreases in spontaneous motor activity. Many of the studies included in this dissertation require animals to be handled prior to the infusion procedure. Therefore, ip LPS was given to both handled and nonhandled animals in order to determine if the effects of ip LPS on spontaneous motor activity would differ depending on whether animals are handled or nonhandled; see handling procedure section in the general methods section.

Methods

Procedure

Female rats were obtained from our group-housed colony room and placed into single housing for approximately three days prior to collecting any data. Following this period, three days (days 1, 2 & 3) of motor activity were recorded during the dark period of each day (1900-0700 hrs.). These three days (days 1, 2 and 3) were averaged and the mean was used to assign the animals to two matched groups: handled and nonhandled rats. During days 4, 5 and 6 one group of animals was handled and the remaining group of animals was left undisturbed (nonhandled). Motor activity was recorded for both groups during the dark period. These three days (days 4, 5 and 6) were averaged and the mean was used to further assign animals to eight matched experimental groups (n=8-12

per group). Each animal in the handled or nonhandled group received one the following ip doses: control (VEH), 5 μg, 10 μg or 20 μg/ml of LPS/kg of body weight. On day seven, ip injections were given just prior to lights out (lights out - 1900 hrs.) and motor activity during the dark period was recorded for 12 hrs. post-injection.

Statistical Analyses

Pre-baseline motor activity (a mean of days 1, 2 & 3) was analyzed by a RM-ANOVA factorial design (12 [hour] X 4 [drug level] X 2 [handled/nonhandled]). Post-injection motor activity also was analyzed by 12 X 4 X 2 RM-ANOVA. Finally, each hour of post-injection motor activity was analyzed by a separate two-way ANOVA (4 X 2).

Results

A RM-ANOVA (hour X drug level X handled/nonhandled) analyzing pre-baseline motor activity yielded a significant main effect of hour ($\underline{F}(11, 858) = 25.76$, $\underline{p} <$ .001]. The remaining main effects of drug level, handled/nonhandled, or any interaction were not statistically significant. This analysis indicated that, while pre-baseline motor activity differed depending on the hour of the dark period, there was no difference in pre-baseline motor activity between any of the groups prior to treatment or between pre-handled vs. pre-nonhandled animals at any hour.

A RM-ANOVA (hour X drug level X handled/nonhandled) analyzing post-injection motor activity yielded a significant main effect of hour [$\underline{F}(11, 858) = 43.12$, $\underline{p} <$ .001], drug level [$\underline{F}(3, 78) = 5.05$, $\underline{p} = .003$] and handled/nonhandled [$\underline{F}(1, 78) = 6.29$, $\underline{p} =$ .010]. All interactions were not statistically significant. The main effect of hour was

derived from a "U-shaped" pattern of motor activity during the dark period that was seen in all drug levels and in both handled and nonhandled rats. The main effect of drug level was derived from the decrease in motor activity seen after an injection of ip LPS. The main effect of handled/nonhandled was derived from the nonhandled animals showing slightly less motor activity than the handled animals (Figure 3 and Figure 4). It is important to note that the three-way interaction (hour X drug level X handled/nonhandled) did not reach statistical significance. This interaction tests whether ip LPS affects handled vs. nonhandled animals differently depending on the dose of LPS given and the hour of the dark period.

Separate two-way ANOVAs (drug level X handled/nonhandled) of post–injection motor activity at each hour of the dark period yielded significant main effects of drug level and handled/nonhandled at different hours (Figure 3 and Figure 4). Specifically, the main effect of drug level was statistically significant at hour 3, 5 and 6 [F(3, 86) = 5.71, 5.30, 4.28, p = .001, .002, .008, hrs. 3, 5 & 6 respectively]. The main effect of drug level was derived from the decrease in motor activity seen after an injection of ip LPS. The main effect of handled/nonhandled was statistically significant at hours 2, 3 and 6 [F(1, 86) = 5.50, 5.20, 5.43, p = .022, .025, .022, hrs. 2, 3, & 6 respectively]. The main effect of handled/nonhandled was derived from the nonhandled animals showing slightly less motor activity than the handled animals. None of the two-way interactions at each hour (drug level X handled/nonhandled) reached statistical significance. A significant two-way interaction would indicate whether the decrease in spontaneous motor activity in

Intraperitoneal VEH or LPS Injections
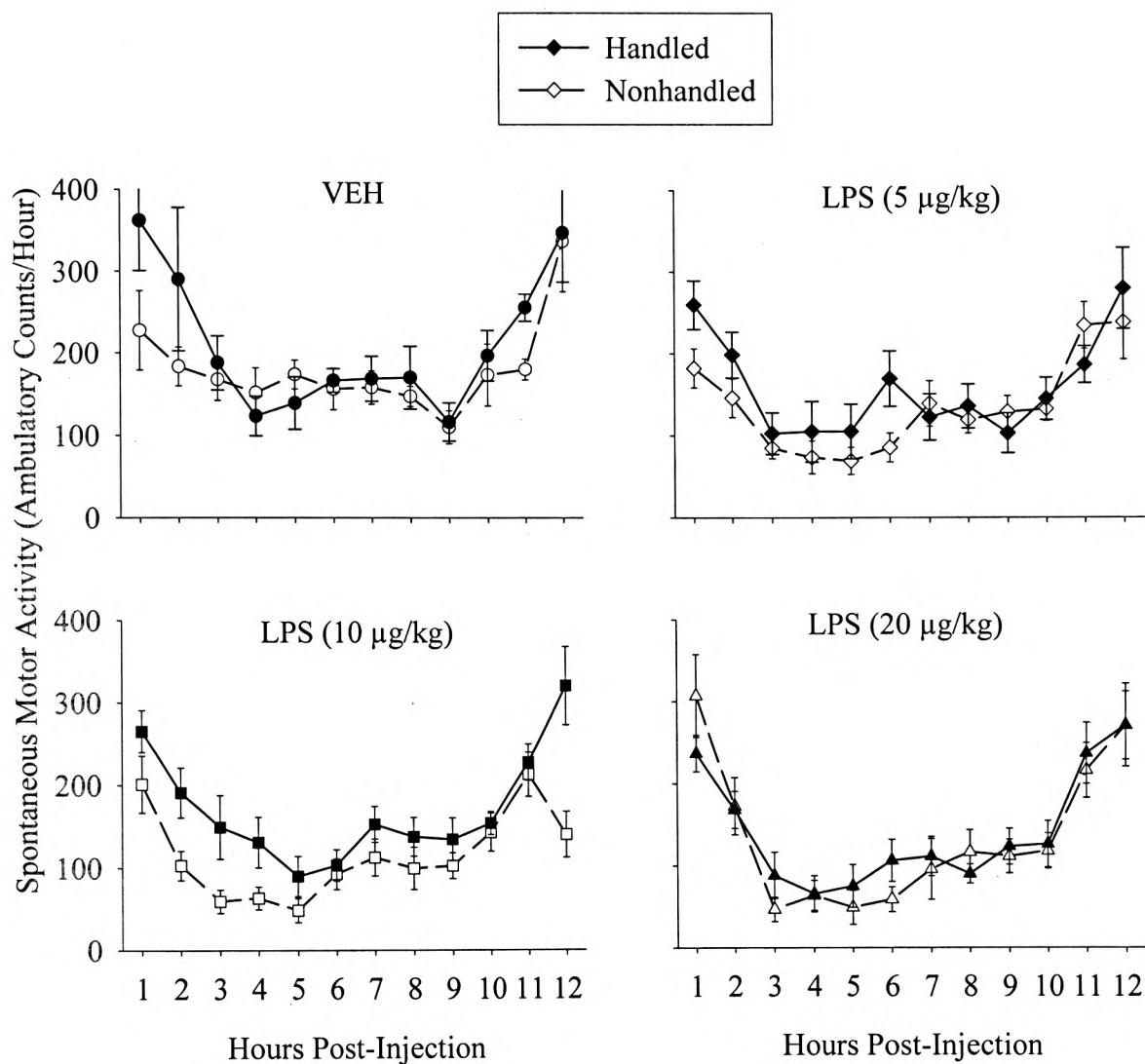
Figure 3. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of handled or nonhandled rats injected with ip VEH or LPS (5, 10 or 20 µg/kg). Graphs show handled and nonhandled animals at each drug level.

Intraperitoneal VEH or LPS Injections
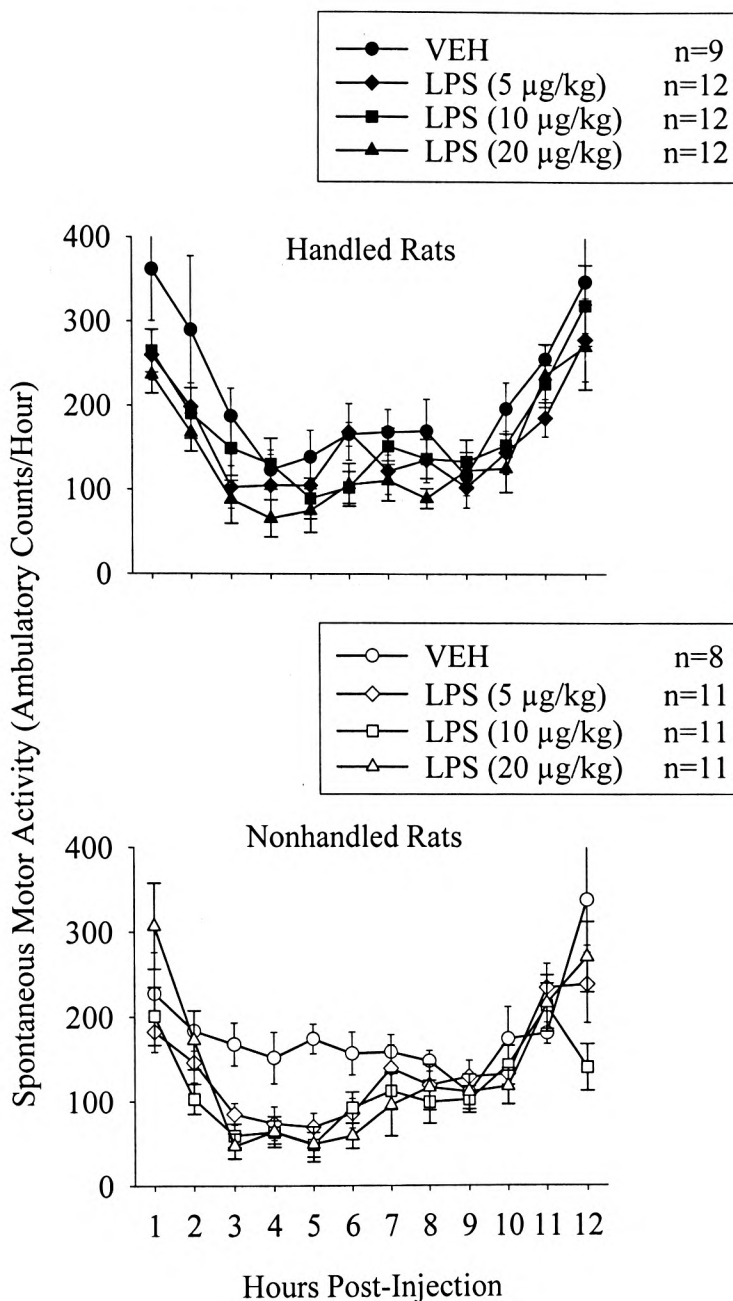
Figure 4. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of handled or nonhandled rats injected with ip VEH or LPS (5, 10 or 20 µg/kg). The top graph shows handled animals at all drug levels and the bottom graph shows nonhandled animals at all drug levels.

response to ip LPS is different depending on whether the animals are handled or nonhandled.

Discussion

Measurement of pre-baseline spontaneous motor activity revealed that the eight groups began the experiment with comparable motor activity. When pre-baseline motor activity was analyzed by a RM-ANOVA (hour X drug level X handled/nonhandled), the significant main effect of hour during the dark period can be described by a "U-shaped" pattern. However, the main effects of drug level and handled/nonhandled, as well as all interactions, were not statistically significant.

A RM-ANOVA (hour X drug level X handled/nonhandled) of post-injection motor activity yielded statistically significant main effects of hour, drug level and handled/nonhandled. The main effects of hour and drug level can be described by a "U-shaped" pattern of motor activity during the dark period and by a decrease in spontaneous motor activity of the animals in response to ip LPS. The main effect of handled/nonhandled can be described by a slightly lower amount of motor activity of nonhandled animals when compared to the handled animals. Although these individual main effects were found, the most important factor in this experiment is the three-way interaction. The three-way interaction tests whether ip LPS affects handled vs. nonhandled animals differently depending on the dose given and the hour of the dark period, and this interaction was not statistically significant.

Although not statistically significant, the trend for ip LPS to decrease spontaneous motor activity occurred in a dose-dependent manner (Figure 3 & Figure 4). Specifically,

VEH did not decrease spontaneous motor activity but the two highest doses of LPS (10 or 20 µg/kg) tended to decrease spontaneous motor activity more effectively than the lowest dose of LPS (5 µg/kg). However, handling did not significantly affect this outcome. The RM-ANOVA (hour X drug level X handled/nonhandled) and the separate two-way ANOVAs (each hour, drug level X handled/nonhandled) of post-injection motor activity indicate that ip LPS at various drug levels did not significantly decrease spontaneous motor activity differently in the handled or nonhandled groups (Figure 3 & Figure 4).

The results from this study suggest that ip LPS does not decrease spontaneous motor activity differently in handled or nonhandled rats. Many of the experiments detailed in this dissertation required animals to be handled prior to the infusion procedures. Therefore, handling animals prior to the infusion should not significantly alter the overall effects in response to LPS and/or "sickness behavior". Specifically, handling animals prior to the infusion of LPS (Exp. 2-Part I) or IL-1 (Exp. 3) should not cause the expected decreases in spontaneous motor activity to be significantly different from what they would be in nonhandled animals.

CHAPTER FOUR

Experiment 2-Part I: LPS Infusions into the LC Region

Rationale

Uncontrollable shock in rats causes an increase in burst-firing of LC neurons and a decrease in motor activity. Similarly, ip LPS or microinfusions of LPS into the LC region also causes an increase in burst-firing of LC neurons, and ip LPS decreases spontaneous motor activity as was seen in Exp. 1. Previous findings have shown that LPS causes the induction of IL-1 in brain microglia for prolonged periods; therefore, prolonged activation of IL-1 receptors can be achieved by infusing LPS into the LC region. If the activation of LC neurons is involved in decreasing spontaneous motor activity and ip LPS has been shown to stimulate IL-1, which causes activation of LC neurons, then an infusion of LPS into the LC region should decrease spontaneous motor activity. Part I of Exp. 2 was to test whether LPS infused into the LC region would cause a decrease in spontaneous motor activity. A range of LPS doses were infused to test whether the effects on motor activity would be dose-dependent. Part II of Exp. 2 describes the diffusion distances of Fluorescein Isothiocyanate LPS (fic-LPS, a fluorescent LPS) from the LC region.

Part I: Methods

Surgical, Spontaneous Motor Activity, and Infusion Procedures

The procedural methods are detailed in the general methods section. Briefly, male rats were obtained from our group-housed colony and single housed in a room where spontaneous motor activity was monitored by a computer-controlled system. After

several days of housing under these conditions, animals underwent surgery. The animals were anesthetized with Halothane and implanted with a bilateral cannula aimed at the LC region (Table 1). Seven to ten days after surgery, spontaneous motor activity was recorded for three days and a mean of these three days was calculated, representing baseline motor activity for the animals. On each of these three days, the animals also were handled. Based on their baseline motor activity, animals were assigned to six matched groups (n=4-12 per group) and infused with CSF or varying doses of LPS (0.1, 0.2, 1, 2, or 4 ng/rat) in 1μl/side of LC, for a total infusion volume of 2 μl/rat. Infusions began one hour prior to lights out (lights out - 19:00 hrs.). After the infusion, the animals were returned to their home cage and motor activity was recorded for 12 hrs. post-infusion during the dark period.

Statistical Analyses

Baseline motor activity was analyzed by a RM-ANOVA factorial design (12 [hour] X 6 [drug level]). Post-infusion motor activity also was analyzed by a 12 X 6 RM-ANOVA. Finally, each hour of post-infusion motor activity was analyzed by a separate one-way ANOVA (1 X 6 [drug level]). Following separate one-way ANOVAs and where appropriate, *post hoc* analyses were conducted by a Dunnett test comparing the LPS infused groups with the CSF-infused group.

Motor activity of animals with cannulae that terminated directly in the LC and animals with cannulae that terminated "near the LC" showed similar effects of LPS on motor activity. Therefore, all of the animals implanted with cannulae aimed at the LC region, with the exception of one animal, were included in the statistical analyses and

data presentation. One animal was omitted due to the large margin by which it missed the LC (0.5-0.6 mm medial/lateral from the LC). Representations of cannulae aimed at the LC region are pictured in Figure 5. Two animals had cannulae placements that were slightly anterior to the LC, as shown on a section reproduction from the stereotaxic atlas (Figure 5, Bregma –8.8 mm). Since the width of the cannula is 0.54 mm, the bevel of the cannulae faces caudally and the placements are sufficiently close to overlap into the first atlas plate that contains the LC (Bregma -9.16 mm), these animals were included in statistical analyses and data presentation. Not all of the animals are represented in Figure 5 because cannulae placements for 12 of the animals were verified and noted only in written form. According to the written notes, all 12 placements were considered to be in the LC region and were included in the statistical analyses and data representation.

Part I: Results

A RM-ANOVA (hour X drug level) analyzing baseline motor activity yielded a significant main effect of hour [$F(11, 451) = 13.82$, $p < .001$]. The remaining main effect of drug level and the two-way interaction were not statistically significant. This analysis indicated that, while baseline motor activity differed depending on the hour of the dark period, there was no difference in baseline motor activity at any hour between any of the groups prior to treatment.

A RM-ANOVA (hour X drug level) analyzing post-infusion motor activity yielded a significant main effect of hour [$F(11, 451) = 26.56$, $p < .001$] and drug level [$F(5, 41) = 4.26$, $p = .003$]. The main effect of hour is derived from a "skewed-U" pattern of motor activity during the dark period that was seen at all drug levels, except for
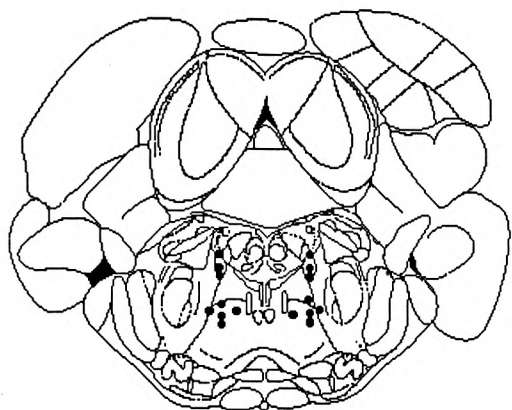
CSF animals; Bregma −9.30 mm
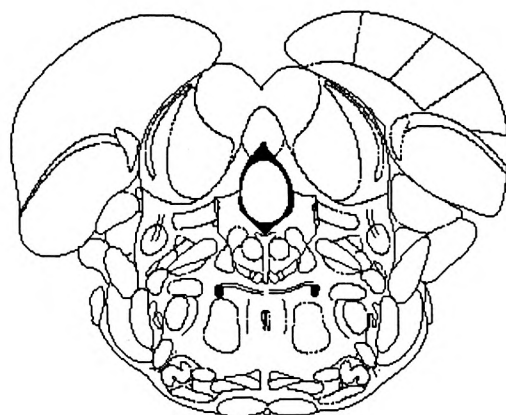
LPS animals; Bregma −8.8 mm
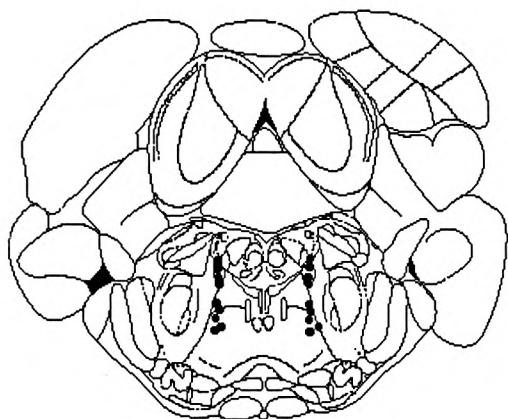
LPS animals; Bregma −9.30 mm
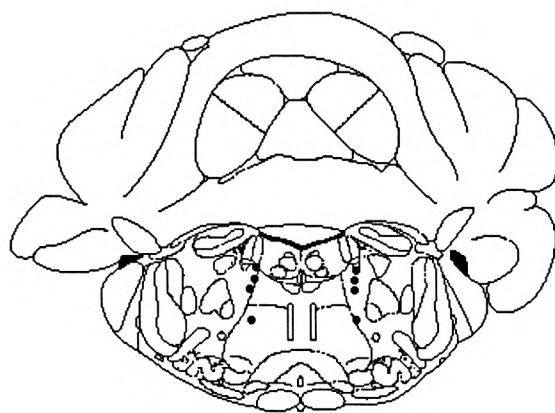
LPS animals; Bregma −9.80 mm

Figure 5. Representative coronal view of cannulae placements that were aimed at the LC region. Top left graph shows represents cannulae placements for animals infused with CSF. The remaining three graphs represent cannulae placements for animals infused with LPS.

[2]From The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1997, New York: Academic Press. Copyright © 1997 by Academic Press. Adapted with permission by Academic Press.

the lowest dose of LPS (0.1 ng LPS/rat) which did not show a decrease in motor activity over the initial hours of the dark period. The main effect of drug level was derived from the decrease in motor activity after the animals received an infusion of LPS into the LC region (Figure 6). The two-way interaction approached statistical significance [F(55, 451) = 1.34, p = .061].

Separate one-way ANOVAs (each hour X drug level) of post-infusion motor activity of the dark period yielded a significant main effect of drug level at hours 3-8 [F(5, 46) = 5.18, 9.69, 6.43, 6.84, 2.89, 3.82; p = .001, ≤ .001, ≤ .001, ≤ .001, = .025, = .006, hrs. 3-8 respectively]. Dunnett's tests performed at each hour comparing post-infusion motor activity of the LPS groups with the CSF group yielded statistically significant differences that were time- and dose-dependent. Specifically, the lowest dose of LPS (0.1 ng/rat) produced increased motor activity at hour eight post-infusion but did not yield a statistically significant difference at any other hour. The second lowest dose of LPS (0.2 ng/rat) decreased motor activity at hours three and four post-infusion. The middle dose of LPS (1 ng/rat) and the two largest doses of LPS (2 and 4 ng/rat) decreased motor activity from hours three to six (Figure 6).

Part I: Discussion

Measurement of baseline spontaneous motor activity revealed that the six groups began the experiment with comparable motor activity. When baseline motor activity was analyzed by a RM-ANOVA (hour X drug level), the significant main effect of hour during the dark period can be described by a "skewed-U" pattern. However, the main effect of drug level or the two-way interaction (hour X drug level) were not statistically
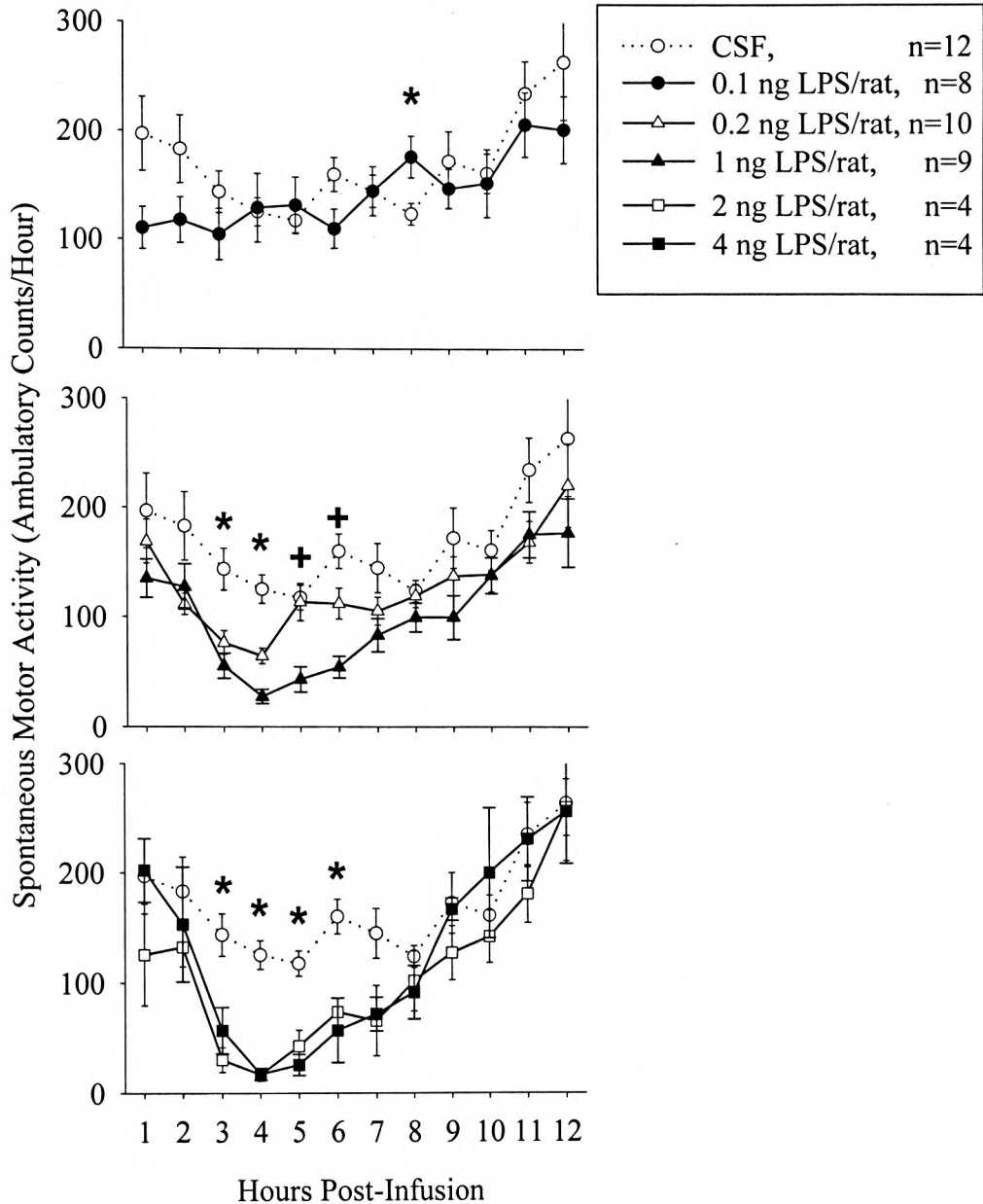
Figure 6. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats infused with CSF or LPS (0.1, 0.2, 1, 2 or 4 ng/rat) into the LC region. Note that the CSF group is included in all of the graphs for easier inspection of the data. The asterisk (*) and plus sign (+) designate a significance level of $p < 0.05$. * = CSF vs. all other doses shown on that section of the figure. + = CSF vs. 1ng/rat of LPS.

significant.

The RM-ANOVA analysis (hour X drug level) of post-infusion motor activity yielded a main effect of hour and drug level, but the two-way interaction term did not reach statistical significance. The main effect of hour indicates that spontaneous motor activity is different across time (hours). The main effect of drug level indicates that LPS infused into the LC region decreases spontaneous motor activity in a dose-dependent manner. The two-way interaction (hour X drug level) was not statistically significant.

Separate Dunnett's tests comparing post-infusion spontaneous motor activity of the various LPS groups with the CSF group at each hour indicate that various doses of LPS decreased spontaneous motor activity (Figure 6). Specifically, the CSF group and the lowest dose of LPS (0.1 ng/rat) group showed similar motor activity across each hour, except for hour eight in which 0.1 ng LPS/rat actually increased activity. The second lowest dose of LPS (0.2 ng/rat) decreased motor activity for two hours while the middle dose (1 ng/rat) and two highest doses of LPS (2 and 4 ng/rat) continued to affect motor activity for a total of four hours. These results indicate that higher doses of LPS infused into the LC region decrease spontaneous motor activity for longer periods than lower doses of LPS.

It is noteworthy that the onset of the decrease in spontaneous motor activity occurs at hour three post-infusion regardless of the dose of infused LPS. Previous findings have shown that LPS causes the induction of IL-1 in the brain. The time delay between the infusion of LPS into the LC region and the onset of the decrease in spontaneous motor activity may be attributable to the time needed for LPS to cause the

induction of IL-1 in the brain. Furthermore, the onset of the decrease in spontaneous motor activity tends to occur in an "all or nothing" manner. This suggests that a threshold level of LPS must be reached for the induction of IL-1 in the brain to cause the decrease in spontaneous motor activity.

Lower doses of LPS infused into the LC region resulted in the animals recovering from the decrease in spontaneous motor activity sooner than animals infused with higher doses of LPS. Specifically, the group receiving the second lowest dose of LPS (0.2 ng/rat) recovered from the decrease in spontaneous motor activity two hours sooner than the groups receiving the three highest doses of LPS (1, 2 and 4 ng/rat). The difference in recovery time from decreased motor activity may be a dose-dependent effect of the LPS, suggesting that higher doses of LPS possibly maintain the induction of IL-1 for longer periods than lower doses of LPS.

Experiment 2-Part II: Fic-LPS Diffusion from the LC Region

Rationale

Part II of Exp. 2 describes the diffusion characteristics of Fluorescein Isothiocyanate LPS (fic-LPS, a fluorescent LPS) from the LC region. This was accomplished by infusing fic-LPS into the LC region and measuring diffusion distances of fic-LPS from the infusion site. Since fic-LPS is LPS that is labeled with a fluorescein, then the diffusion characteristics for both compounds should be similar.

Part II: Methods

Surgical, Spontaneous Motor Activity, and Infusion Procedures

Surgical and infusion procedural methods are similar to those described in Part I of Exp. 2 and are detailed in the general methods section. Spontaneous motor activity procedural methods are similar as in Part I of Exp. 2 except for slight modifications that were incorporated into the new computer software and are detailed in the general methods section.

Briefly, male rats were obtained from our group-housed colony and single housed in a room where spontaneous motor activity was monitored by a computer-controlled system. After several days of housing under these conditions, animals underwent surgery. The animals were anesthetized and implanted with a bilateral cannula aimed at the LC region (Table 1). Seven to ten days after surgery, spontaneous motor activity was recorded for two days and a mean of these two days was calculated, representing baseline motor activity for the animals. On each of these two days, the animals also were handled. Based on their baseline motor activity, animals were assigned to two matched conditions

(n=2 per condition) that would be killed at different times after the infusion. In one condition the animals were killed one hour post-infusion (referred to as the "1-hour condition") and the other condition the animals were killed 22 hours post-infusion (referred to as the "22-hour condition). Both conditions were infused with 4 ng/rat of fic-LPS in a total volume of 2 µl/rat. Infusions began one hour prior to lights out (lights out - 19:00 hrs.). After the infusion, the animals were returned to their home cage and motor activity was recorded for 12 hrs. post-infusion during the dark period, only for the 22-hour condition.

Histology

Animals were anesthetized with Halothane and killed at 1 or 22 hours post-infusion. Fresh brain tissue was quickly removed and placed on dry ice. The LC region and ±2 mm were cut at 50 µm on a cryostat and alternating sections were mounted on clean slides. The sections that contained the cannula tract were viewed under a fluorescent microscope and the beginning, the end, and the center of the infusion were identified. The coronal section with the center of the infusion was photographed at a magnification of 25 by 1X, and a three dimensional diffusion distance of fic-LPS was measured. The anterior-posterior distance covered by fic-LPS was calculated by the number of coronal sections in which fic-LPS was visible (50 µm/section). The medial-lateral and dorsal-ventral distances were calculated by measuring the width and height of visible fic-LPS using the line tool in Image Pro Express (Version 4).

Part II: Results

Baseline motor activity during the dark period is plotted with its respective post-infusion motor activity for each animal and are shown in Figure 7. An inspection of the graphs for both animals show a pronounced decrease in motor activity after the animals were infused with 4 ng/rat of fic-LPS into the LC region. The decrease in motor activity is clearly seen when post-infusion motor activity is plotted against each animal's own baseline motor activity. An infusion in both animals of fic-LPS decreased motor activity from hours 2-10 post-infusion.

Photographs of coronal sections showing the spread and shape of fic-LPS diffusion are shown in Figure 8. The 1-hour condition showed fic-LPS in both sides of the LC region. The diffusion distances of fic-LPS for the left and right sides were measured and an average was calculated for anterior-posterior, medial-lateral and dorsal-ventral (Figure 8, left side of page). For one animal, average distances of diffusion from the center of the infusion site of fic-LPS were 700 μm anterior-posterior, 210 μm medial-lateral and 480 μm dorsal-ventral. For the other animal, average distances of diffusion from the center of the infusion site of fic-LPS were 250 μm anterior-posterior, 185 μm medial-lateral and 295 μm dorsal-ventral (stained photographs not shown).

Diffusion distances of fic-LPS for the 22-hour condition were much smaller than the 1-hour condition. For one animal in the 22-hour condition, the fic-LPS infusion only of the left side covered a distance of 100 μm anterior-posterior, 190 μm medial-lateral and 180 μm dorsal-ventral (Figure 8, right side of page). The infusion on the right side of the same animal was only visible in one section. The remaining animal in the 22-hour

Fluorescein Isothiocyanate LPS Infusions into the LC Region
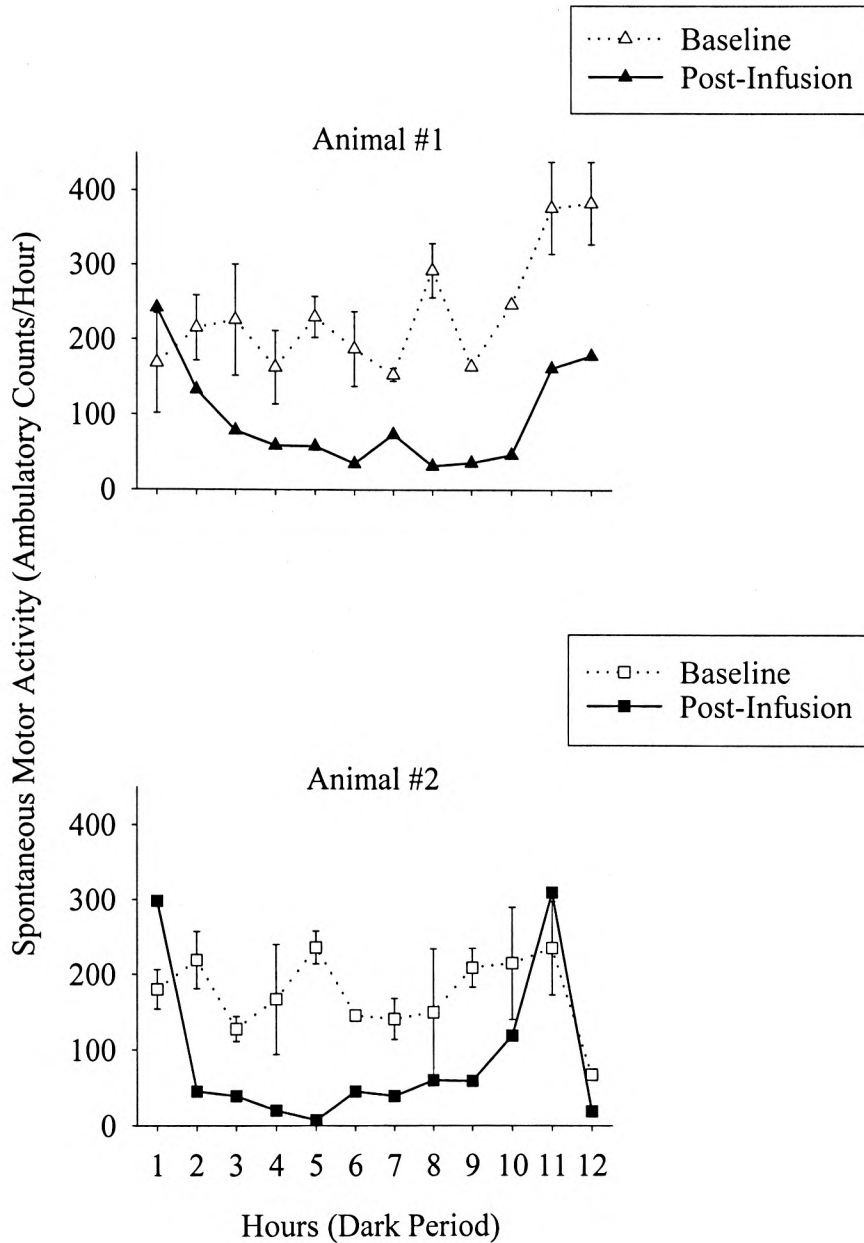
Figure 7. Ambulatory counts/hour (dark period spontaneous motor activity) of individual rats not infused (baseline) or infused with Fluorescein Isothiocyanate LPS (4 ng/rat) into the LC region and killed 22 hours post-infusion.

Flourescein Isothiocyanate LPS Infusions into the LC Region

Bregma −10.30 mm
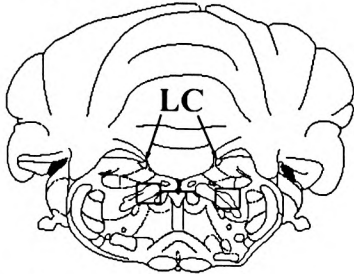
Bregma − 9.80 mm

(1-hour condition)
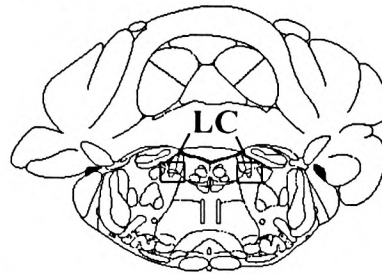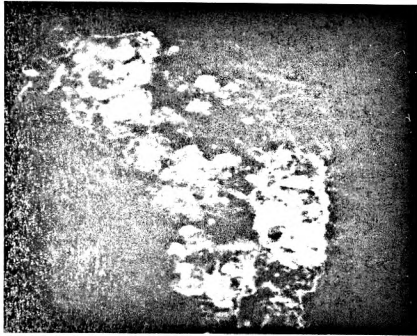
(22-hour condition)

Left (1-hour condition)

Left (22-hour condition)

Right (1-hour condition)
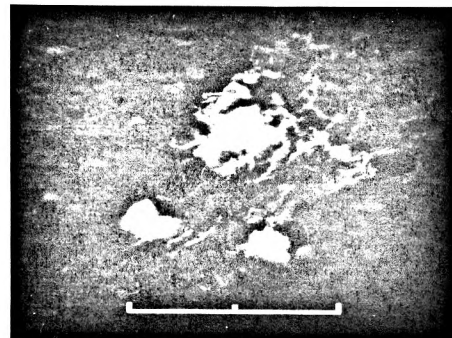
Right (22-hour condition)

Figure 8.   Stained photographs showing the center of the infusion site for both left and right sides of the LC region for individual animals infused with 4 ng/rat of fic-LPS.
The two graphs shown at the top are section reproductions from the stereotaxic atlas. Arrows labeled LC point to the LC region.   Rectangular boxes correspond to the fields shown in the photographs directly below.   The full length of the scale shown in the bottom right photograph equals 200 μm and applies to all photographs on the page.
[4]From The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1997, New York: Academic Press.   Copyright © 1997 by Academic Press.   Adapted with permission by Academic Press.

condition showed fic-LPS only in a very small area on the left side and none on the right side of the LC region (stained photographs not shown).

Part II: Discussion

Fic-LPS into the LC region decreased spontaneous motor activity (Figure 7) of the 22-hour condition. These decreases are similar to the decreases seen after an infusion of LPS into the LC region (Exp. 2-Part I). The decreases in spontaneous motor activity began two hours post-infusion of fic-LPS and the effects continued for a total of nine hours. Similar decreases would have been expected from the animals that also were infused with fic-LPS but were killed one hour post-infusion.

Visible fluorescence of fic-LPS in the 22-hour condition was much smaller than the fluorescence of fic-LPS in the 1-hour condition. The indication that the fic-LPS infused into the 22-hour condition covered a smaller distance than in the 1-hour condition may be due to the fic-LPS diffusing away from the original site of infusion. The prolonged time allotted for the fic-LPS to diffuse in the 22-hour condition could result in less fluorescence at the infusion site. However, visible fluorescence of fic-LPS in both conditions remained within 500 microns anterior-posterior, medial-lateral or dorsal-medial from the center of the infusion site. This suggests that an infusion at a distance greater than 0.5 mm from the LC (in a three dimensional plane) could be used to test the specificity of the LC in responding to a drug as was done in Exp. 4 and Exp. 6.

CHAPTER FIVE

Experiment 3: IL-1β Infusions into the LC Region

Rationale

Exposure of rats to uncontrollable shock causes an increase in burst-firing of LC neurons, resulting in a decrease in motor activity. Similarly, ip LPS increases burst-firing of LC neurons and decreases spontaneous motor activity. As stated in the introduction, the increase in burst-firing of LC neurons after ip LPS appears to be mediated by IL-1 in the brain. Therefore, the purpose of Exp. 3 was to test whether IL-1β microinfused into the LC region would decrease spontaneous motor activity as seen after ip LPS, thereby suggesting that ip LPS acts by stimulating IL-1 in the LC region. This was accomplished by infusing a range of IL-1β doses into the LC region to determine if IL-1β causes dose-dependent decreases in spontaneous motor activity. The IL-1β dose-response curve also will determine the smallest effective dose that reliably results in a decrease in spontaneous motor activity. The smallest effective dose of IL-1β was then infused into the LC region and LC surrounding regions to determine specificity of IL-1 action (Exp. 4) and to measure changes in general behaviors that are produced by an infusion IL-1 into the LC region (Exp. 5).

Methods

Surgical, Spontaneous Motor Activity, and Infusion Procedures

The procedural methods are similar to Exp. 2 and are detailed in the general methods section. Briefly, male rats were obtained from our group-housed colony and single housed in a room where spontaneous motor activity was monitored by a computer-

controlled system. After several days of housing under these conditions, animals underwent surgery. The animals were anesthetized with Halothane and implanted with a bilateral cannula aimed at the LC (Table 1). Seven to ten days after surgery, spontaneous motor activity was recorded for three days and a mean of these three days was calculated, representing baseline motor activity for the animals. On each of these three days, the animals also were handled. Based on their baseline motor activity, animals were assigned to six matched groups (n=3-7 per group) and infused with CSF or varying doses of IL-1β (0.1, 0.2, 0.4, 4.0, 40 ng/rat) in 1 µl/side of LC, for a total infusion volume of 2 µl/rat. Infusions began 30 min. prior to lights out (light out − 19:00 hrs.). After the infusion, the animals were returned to their home cage and motor activity was recorded for 12 hrs. post-infusion during the dark period.

Statistical Analyses

Baseline motor activity was analyzed by a RM-ANOVA factorial design (12 [hour] X 6 [drug level]). Post-infusion motor activity was analyzed by a 12 X 6 RM-ANOVA. Finally, each hour of post-infusion motor activity was analyzed by a separate one-way ANOVA (1 X 6 [drug level]). Following each of the separate one-way ANOVAs at each hour and where appropriate, *post hoc* analyses were conducted by a Dunnett test comparing the IL-1-infused groups with the CSF-infused group.

<div align="center">Results</div>

A RM-ANOVA (hour X drug level) analyzing baseline motor activity yielded a significant main effect of hour ($\underline{F}$(11, 275) = 23.42, $\underline{p}$ < .001]. The remaining main effect of drug level and the two-way interaction were not statistically significant. This analysis

indicated that, while baseline motor activity differed depending on the hour of the dark period, there was no difference in baseline motor activity at any hour between any of the groups prior to treatment.

A RM-ANOVA (hour X drug level) analyzing post-infusion motor activity yielded a main effect of hour [$F_{(11, 275)}$ = 16.41, p < .001] and drug level [$F_{(5, 25)}$ = 3.6, p = .013]. The main effect of hour was derived from a rise in motor activity toward the end of the dark period. The main effect of drug level was clearly derived from a decrease in motor activity after an infusion of IL-1β into the LC region. The two-way interaction (hour X drug level) was not statistically significant.

Separate one-way ANOVAs (each hour X drug level) of post-infusion motor activity of the dark period yielded a significant main effect of drug level from hours 3-7 [$F_{(5, 30)}$ = 5.28, 6.78, 3.62, 2.71, 6.52; p = .002, ≤ .001, = .013, = .044, = .001, hrs. 3-7 respectively]. Dunnett's tests performed at each hour comparing post-infusion motor activity of the IL-1β-infused groups with the CSF-infused group yielded statistically significant differences that were time- and dose-dependent (Figure 9). Specifically, the two lowest doses of IL-1β (0.1 and 0.2 ng/rat) did not significantly decrease motor activity at any hour. The middle dose of IL-1β (0.4 ng/rat) significantly decreased motor activity at hour four and the second highest dose of IL-1β (4 ng/rat) decreased motor activity at hours 3-5. The highest dose of IL-1β (40 ng/rat) decreased motor activity at hours 3-5 and hour 7 (Figure 9).
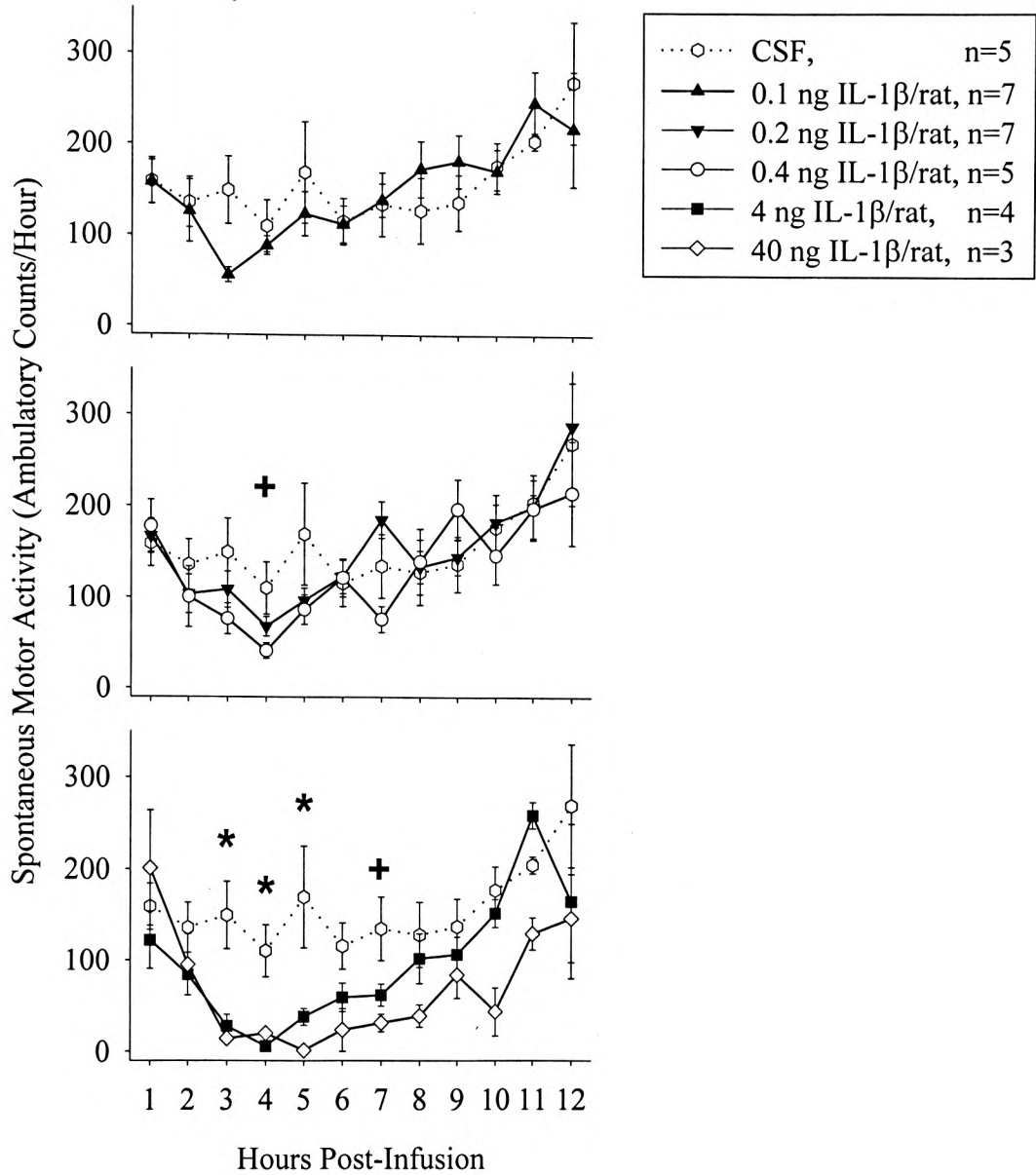
Figure 9. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats infused with CSF or IL-1β (0.1, 0.2, 0.4, 4.0 or 40 ng/rat) into the LC region.
Note that the CSF group is included in all of the graphs for easier inspection of the data. The asterisk (*) and plus sign (+) designate a significance level of $p < 0.05$. * = CSF vs. all other doses shown on that section of the figure. + = CSF vs. the highest dose shown on that section of the figure.

Discussion

Measurement of baseline spontaneous motor activity revealed that the six groups began the experiment with comparable motor activity. When baseline motor activity was analyzed by a RM-ANOVA (hour X drug level), the significant main effect of hour during the dark period can be described by a rise in motor activity toward the end of the dark period. However, the main effect of drug level and the two-way interaction (hour X drug level) were not statistically significant.

The RM-ANOVA analysis (hour X drug level) of post-infusion motor activity yielded a main effect of hour and drug level. The main effect of hour indicates that spontaneous motor activity is different across time (hours). The main effect of drug level indicates that IL-1β infused into the LC region decreases spontaneous motor activity in a dose-dependent manner. The two-way interaction (hour X drug level) was not statistically significant.

Separate Dunnett's tests comparing post-infusion spontaneous motor activity of the various IL-1β-infused groups with the CSF-infused group at each hour indicate that various doses of IL-1β decreased spontaneous motor activity in a time- and dose-dependent manner (Figure 9). Specifically, the two lowest doses of IL-1β did not significantly decrease spontaneous motor activity. The middle dose of IL-1β (0.4 ng/rat) decreased spontaneous motor activity at the third hour post-infusion. The two highest doses of IL-1β (4, 40 ng/rat) decreased spontaneous motor activity for a total of three and four hours respectively and did so in a more pronounced manner than the middle dose of infused IL-1β (0.4 ng/rat). These results indicate that higher doses of IL-1β infused into

the LC region decrease spontaneous motor activity for longer periods than lower doses of IL-1β.

A secondary purpose of this experiment was to determine the smallest effective dose of IL-1β that resulted in a reliable decrease in spontaneous motor activity; therefore, a range of IL-1β doses were infused into the LC region. When separate one-way ANOVAs (each hour X drug level) of post-infusion motor activity were followed by Dunnett's tests the smallest dose of IL-1β that was significantly different from the CSF-infused group was the 0.4 ng of IL-1β /rat. Statistical analyses indicate that the 0.4 ng of IL-1β /rat is the smallest effective dose of IL-1β.

In addition to the information obtained from the statistical analyses described above, supporting evidence can be obtained from separately reviewing the data at each drug level. Each section of Figure 10 shows post-infusion motor activity with its corresponding baseline motor activity at each drug level. Inspection of the graphs shows that the two lowest doses of IL-1β (0.1, 0.2 ng/rat) decreased spontaneous motor activity for approximately two hours during the early portion of the dark period. The middle dose of IL-1β (0.4 ng/rat) decreased motor activity for what appeared to be five hours across the dark period. The two highest doses of IL-1β (4 and 40 ng/rat) decreased motor activity for approximately seven and 11 hours across the dark period, respectively. Furthermore, the graphs show that the 0.4 ng IL-1β/rat group affects motor activity for a longer period of time and the effect is more pronounced than the two lowest doses of IL-1β (0.1, 0.2 ng/rat). The effects of the 4 ng IL-1β/rat are even longer and more

CSF or IL-1β Infusions into the LC Region
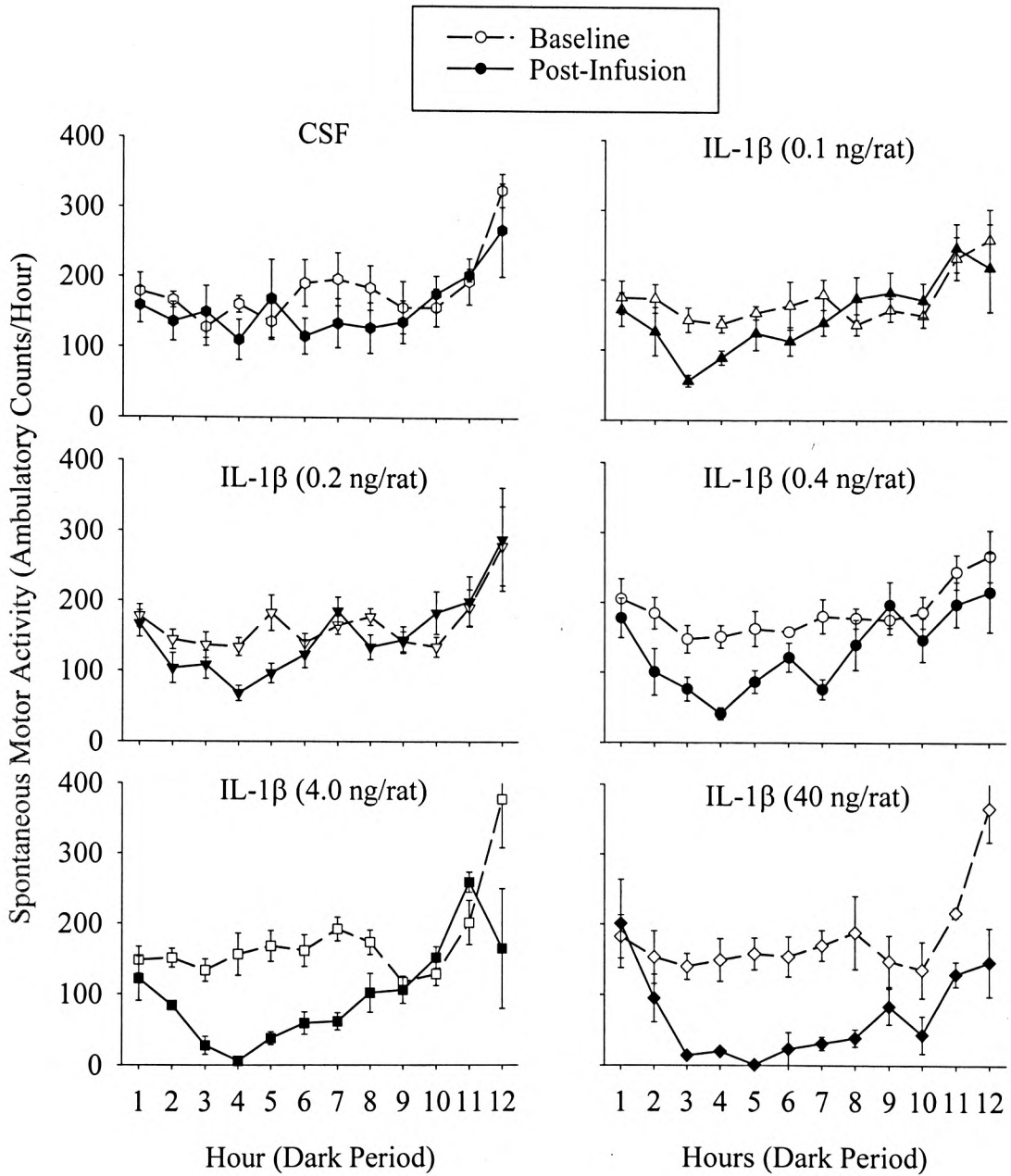
Figure 10.   Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats that have not been infused (baseline) and of rats infused (post-infusion) with CSF or IL-1β (0.1, 0.2, 0.4, 4 or 40 ng/rat) into the LC region.

pronounced than the 0.4 ng IL-1β/rat group. Finally, the effects of the highest dose of IL-1β (40 ng/rat) extend across an 11 hour period and are even more robust than any other dose of infused IL-1β. The results obtained from both the statistical analyses and visually inspecting the data at each drug level indicate that the 0.4 ng of IL-1β /rat is the smallest effective dose of IL-1β; therefore, this dose of IL-1β (0.4 ng/rat) was chosen to conduct Exp. 4 and Exp. 5.

CHAPTER SIX

Experiment 4. IL-1β Infusions into the LC and LC Surrounding Regions

Rationale

Exp. 4 tested whether the predicted decrease in spontaneous motor activity after an infusion of IL-1β into the LC region is specific to the LC. This was done by assessing whether the decrease in motor activity would occur only if IL-1β were infused into the LC region and would not occur if IL-1β were infused into LC surrounding regions. The dose of IL-1β used in this experiment was based on results from Exp. 3 in which 0.4 ng of IL-1β/rat infused into the LC region caused a statistically significant decrease in spontaneous motor activity.

Originally, six original brain regions were chosen for this experiment. The LC was chosen as the main region of focus. The next four regions were chosen based on their proximity to the LC and for the potential of these regions to be affected when IL-1β is infused into the LC region. The four regions are as follows: 1) lateral to the LC (l-LC), 2) medial to the LC (m-LC), 3) the aqueduct (AQ), and 4) the fourth ventricle (4V). The two latter regions are part of the ventricular system and an infusion of IL-1β into the LC region could result in the diffusion of IL-1β into the ventricular system. The sixth region, the area postrema/nucleus of the solitary tract (AP/NST) was chosen based on the potential of the AP to receive the LPS signal when an injection of ip LPS is given. The AP is a circumventricular organ that projects to the LC via the NST (Luppi et al., 1995). Circumventricular organs lack the BBB and the ip LPS signal could reach the brain via

the AP. It is possible that the AP/NST functions concurrently with the LC to cause a decrease in spontaneous motor activity after ip LPS.

After the completion of the experiment, a seventh brain region was added. Cannula placements verified that some cannulae were dorsomedial to the LC and terminated in the fourth ventricle. A representative cannula is shown in Figure 11.
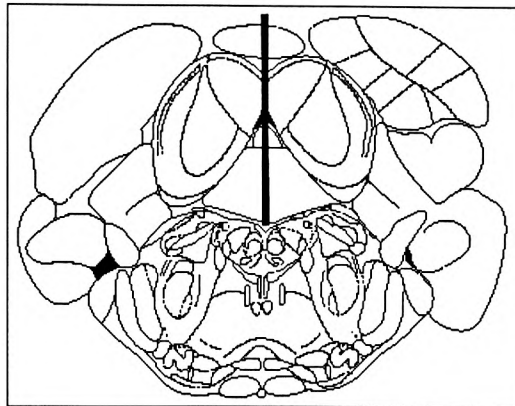
Figure 11. A representative coronal view (Bregma − 9.30 mm) of a cannula terminating dorsomedial to the LC (dm-LC).
[3]From The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1997, New York: Academic Press. Copyright © 1997 by Academic Press. Adapted with permission by Academic Press.

Methods

Surgical Procedure

The procedural methods are similar to Exps. 2 and 3 and are detailed in the general methods section. Briefly, male rats were obtained from our group-housed colony and single housed in a room where spontaneous motor activity was monitored by a computer-controlled system. After several days of housing under these conditions, animals underwent surgery. The animals were anesthetized with Halothane and implanted with a bilateral cannula aimed at the LC or l-LC or a unilateral cannula aimed at the m-LC, AQ, 4V, or the AP/NST [n=4-9 per group, (Table 1)]. The dm-LC group was added when cannulae placement verification confirmed that several animals had cannulae that were dorsomedial to the LC and terminated in the fourth ventricle.

The DV coordinate (Table 1) for the AQ is variable (approximately -5.0 DV) because permanent placement of the AQ cannula is determined at the time of surgery by the presence of CSF in the cannula. CSF rising through the cannula is indicative that the cannula is correctly positioned within the ventricular system. After implanting and securing the cannula, an obturator (a thin piece of nylon) was placed inside the cannula and the cannula was sealed with an electrical pin connector. Although some animals were implanted with a cannula aimed at the 4V, the pressure exerted by endogenous CSF at that point is generally not sufficient to cause the CSF to flow upward through the cannula. Therefore, cannulae aimed at the 4V have predetermined coordinates (Table 1).

<u>Spontaneous Motor Activity and Infusion Procedures</u>

Seven to ten days after surgery, spontaneous motor activity was recorded. The procedural methods are similar to Exp. 2 and 3 and are detailed in the general methods section. Briefly, spontaneous motor activity was recorded for three days and a mean of these three days was calculated, representing baseline motor activity for the animals. On each of these three days, the animals also were handled. Based on cannula location, animals were assigned to six groups (n=4-9 per group). As stated earlier, a seventh group was added based on cannula location and after completing the experiment. All of the animals were infused with 0.4 ng IL-1β/rat in a total infusion volume of 2 μl. Infusions began one hour prior to lights out (lights out – 19:00 hrs.). After the infusion, the animals were returned to their home cage and motor activity was recorded for 12 hrs. post-infusion during the dark period.

<u>Statistical Analyses</u>

Baseline motor activity was analyzed by a RM-ANOVA factorial design (12 [hour] X 7 [brain region]). Post-infusion motor activity was also analyzed by a similar 12 X 7 RM-ANOVA. Finally, each hour of post-infusion motor activity was analyzed by a separate one-way ANOVA (1 X 7).

<div align="center"><u>Results</u></div>

A RM-ANOVA (hour X brain region) analyzing baseline motor activity yielded a main effect of hour ($\underline{F}(11, 473) = 17.16$, $\underline{p} < .001$]. The remaining main effect of brain region and the two-way interaction were not statistically significant. This analysis indicated that, while baseline motor activity differed depending on the hour of the dark

period, there was no difference in baseline motor activity at any hour between any of the brain region groups.

A RM-ANOVA (hour X brain region) analyzing post-infusion motor activity yielded a main effect of hour [F(11, 473) = 20.63, p < .001] which was derived from a "wide-U" pattern of activity during the dark period (Figure 12). The remaining main effect of brain region and the two-way interaction were not statistically significant. A significant two-way interaction would indicate that IL-1β decreases spontaneous motor activity in a time-dependent and brain region-dependent manner. Separate one-way ANOVAs (each hour X brain region) of post-infusion motor activity of IL-1β also did not yield statistically significant effects at any hour.

<div align="center">Discussion</div>

Measurement of baseline spontaneous motor activity revealed that the seven groups began the experiment with comparable motor activity. When baseline motor activity was analyzed by a RM-ANOVA (hour X brain region), the significant main effect of hour during the dark period can be described by a "wide-U" pattern. However, the main effect of drug level and the two-way interaction were not statistically significant.

The RM-ANOVA analysis (hour X brain region) of post-infusion motor activity only yielded a main effect of hour. The main effect of hour indicates that spontaneous motor activity is different across time (hours), and was derived from the "wide-U" pattern of motor activity during the dark period. Although a main effect of hour was found, the

Infusions of IL-1β into the LC and LC Surrounding Regions
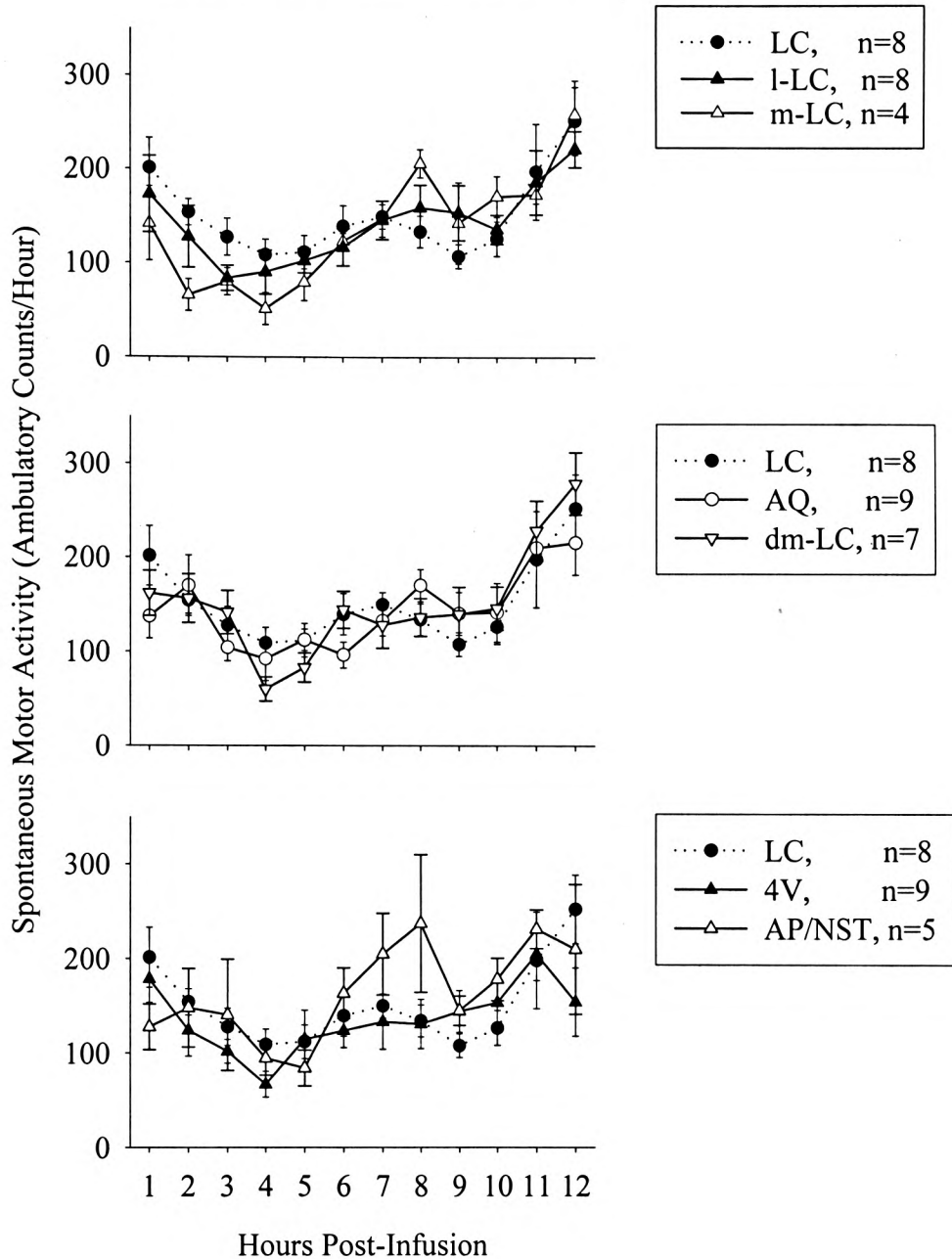
Figure 12.   Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats infused with IL-1β (0.4 ng/rat) into the LC region and LC surrounding regions.
Note that the LC group is included in all of the graphs for easier inspection of the data.

most important factor in this experiment is the two-way interaction, and it was not statistically significant. A statistically significant two-way interaction with respect to post-infusion motor activity would indicate that IL-1β decreased spontaneous motor activity differently across the hours of the dark period depending on the brain region infused. This is confirmed by separate one-way ANOVAs at each hour.

As the statistical analyses described in the previous paragraph indicate, spontaneous motor activity in response to an infusion of IL-1β was similar across all brain regions including the LC region. Inspection of Figure 12 shows that IL-1β infused into all of these brain regions appears to have decreased motor activity beginning at hour two and continuing through hour five post-infusion. To assess whether motor activity was indeed decreased by an infusion of IL-1β, a new RM-ANOVA was conducted adding a control group that showed normal activity (i.e., no decrease) – the group from Exp. 3 that received CSF into the LC region. The new RM-ANOVA (12 [hour] X 8 groups [7 brain regions and the LC-CSF group]) analyzing post-infusion motor activity yielded a main effect of hour [$\underline{F}(11, 517) = 21.14$, $\underline{p} < .001$]. However, the remaining main effect of groups and the two-way interaction were not statistically significant. Additionally, separate one-way ANOVAs (each hour X groups [7 brain regions and the LC-CSF group]) also did not show a statistically significant effect of group at any hour. These results indicate that, in the present experiment, infusion of IL-1β did not produce a statistically significant decrease in spontaneous motor activity. Based on the results that were obtained in Exp. 3, 0.4 ng of IL-1β/rat was chosen for use in the study described here because this was found to be the lowest dose of IL-1β that caused a statistically

significant decrease in motor activity. It is unclear why 0.4 ng of IL-1β/rat did not yield a statistically significant decrease in motor activity in the present experiment, at least when infused into the LC region.

CHAPTER SEVEN

Experiment 5: Effects of IL-1β Infusions into the LC Region on Various Behaviors

Rationale

Experiment 5 was to measure changes in food and water intake, home-cage behaviors (posture, grooming, stretching, digging, etc.), and swim-test behaviors (struggling and floating) in addition to spontaneous motor activity in response to IL-1β infused into the LC region. Procedural methods for food and water intake, home-cage behaviors, and the swim test are detailed in appropriately labeled methods sections below. See Appendix B for a complete list of home-cage behaviors.

Procedurally, three sets of animals were used in this experiment. The first set of animals received 0.4 ng IL-1β/rat or CSF, and spontaneous motor activity, food and water intake, home-cage behaviors, and activity in a swim test (struggling and floating) were measured. When the first set of animals did not show any effects of 0.4 ng/rat of IL-1β either on spontaneous activity or other behaviors, the experiment was repeated except that a higher dose of IL-1β was given. This second set of animals was infused with 1.6 ng IL-1β/rat or CSF, and spontaneous motor activity, food and water intake, and home-cage behaviors were measured. The final set of animals was infused with 1.6 ng IL-1β/rat or CSF, and activity in a swim test (struggling and floating) was measured.

The first set of animals was infused into the LC region with the smallest dose of IL-1β that effectively decreased spontaneous motor activity in Exp. 3. Animals were infused into the LC region with CSF or 0.4 ng/rat of IL-1β, and motor activity, food and water intake, and home-cage behaviors were measured. One week later, the same

animals were reinfused and swim tested. IL-1β infused into the LC region should not affect behavior for more than 10-12 hrs; therefore, one week between the first infusion (used to test motor activity, food and water intake and home-cage behaviors) and the second infusion (used for the swim test) seemed to be an appropriate waiting period. At the time of the second infusion, a partial counterbalancing procedure was used, based on the drug previously infused, to assign animals to two matched groups: CSF group or IL-1β group. The second set of animals was infused with CSF or 1.6 ng/rat of IL-1β and motor activity, food and water intake, and home-cage behaviors were measured. The final set of animals was infused with CSF or 1.6 ng/rat of IL-1β, and swim-test behaviors were measured. Detailed information is provided below.

Methods

Animals

Animals for 0.4 ng IL-1β/rat and its respective CSF group

Naive male rats were selected from our group-housed colony (lights on from 0700-1900 hrs.) and were transferred into an inverse light: dark period (lights on 2400-1200 hrs.). After remaining group-housed for three to four weeks, animals were single housed in a room maintained on an inverse light: dark period that was dedicated to monitoring of motor activity. Motor activity, food and water intake, home-cage behaviors and swim-test behaviors were measured.

Animals for 1.6 ng IL-1β/rat and its respective CSF group

Naive male rats were selected from our group-housed colony and treated as the 0.4 ng IL-1β/rat and the CSF group above. For the rats of this set, only motor activity,

food and water intake, and home-cage behaviors were measured. Another set of naive male rats was selected from our group-housed colony and was used to measure swim-test behaviors. The rats used in the swim-test measurements were placed into single housing but did not undergo a change into an inverse light: dark period; however, swim-test behaviors were measured during the dark period. The animals remained single housed in the colony room (lights on from 0700-1900 hrs.) for approximately three days prior to the surgical procedure.

General Procedures

Detailed procedural methods for food and water intake, home-cage behavior observation and the swim test are detailed in the labeled sections below. The procedural methods for spontaneous motor activity are similar as in previous experiments except for slight modifications that were incorporated into the new computer software and are detailed in the general methods section. Surgical and infusion procedural methods are similar to those described in previous experiments and are also detailed in the general methods section; therefore, procedural methods are briefly discussed. Once animals were placed into single housing, they remained under these housing conditions for several days prior to undergoing surgery. The animals were anesthetized with Halothane and implanted with a bilateral cannula aimed at the LC (Table 1). Seven to ten days after surgery, spontaneous motor activity, food and water intake were recorded for three days and a mean of these three days was calculated for each variable, representing baseline motor activity, food and water intake for the animals. On each of these three days, the animals also were handled. Based on their motor activity, food and water intake, the first

and second set of animals were assigned to matched groups. The first set of animals was assigned to two matched groups (n=3 per group), infused with CSF or IL-1β (0.4 ng/rat) and spontaneous motor activity, food and water, home-cage behaviors and activity in a swim test were measured. The second set of animals was assigned to two matched groups (n=3 per group), infused with CSF or IL-1β (1.6 ng/rat) and only motor activity, food and water intake and home-cage behaviors were measured. The third set of animals was randomly assigned to two groups (n=3-4 per group), infused with CSF or IL-1β (1.6 ng/rat) and activity in a swim test was measured. For all animals, CSF or IL-1β were infused in an alternating order. Infusions began one hour prior to lights out. After the infusion, the animals were returned to their home cage. Home-cage behaviors were observed for a total of two hours during the dark period. Motor activity was recorded for 12 hrs. post-infusion during the dark period. Food and water intake were recorded during the following light period. Finally, activity in the swim test (struggling and floating) was measured.

Food and Water Intake

Food and water were provided *ad libitum* and were measured daily, to the nearest 0.01 g and 1 g respectively, for a three-day period. These three days were averaged and a mean intake baseline was calculated. Food and water intake also were recorded approximately 20 hours post-infusion.

Home-Cage Behaviors

Home-cage behaviors are detailed in Appendix B. Home-cage behaviors were observed for a total of two hours from hour four to hour six post-infusion. The room was

illuminated by red light and the duration of each behavior was recorded using a strip recorder. An investigator sat in the center of the room and timed active and nonactive behaviors for 4 min. per animal. The order in which the animals were observed followed the same order in which they were infused (alternating between CSF and IL-1β).

A strip recorder loaded with block graph paper and advancing at 6 cm/min. was used to record the appropriate behavior for each animal observed. Specifically, as the graph paper advanced, a line was drawn and labeled every time a new behavior would occur. Some of the behaviors that were recorded were as follows: Awake or asleep state, curled, sitting, digging, eating, grooming, motor behavior, standing, rearing, and stretching. The lines indicate the beginning and end of a particular behavior and the distance between the lines were translated into time spent expressing that behavior.

Each animal was observed for 4 min. until all six animals had been observed. This procedure was repeated three more times, in the same order (totaling four times per animal), until all animals had been observed for a total of 16 min. each. One minute was allotted between animals for proper animal identification and to reset the timer (allotting 5 min. per animal/session for a total of 4 sessions). The above procedure allowed the investigator to observe home-cage behaviors for four-4 min. sampling periods, totaling a two hour block, during hours four to six post-infusion.

Swim-Test Procedure

Animals that were swim tested in the "0.4 ng IL-1β/rat and CSF set" had been infused one week earlier and motor activity, food and water intake and home-cage behaviors had been measured. Animals that were swim tested in the "1.6 ng IL-1β/rat

and CSF set" were naive. Procedural methods for the swim test for both sets of animals were the same and are detailed below.

The swim-test procedure used in our laboratory is a modification of a swim test initially described by (Porsolt et al., 1977). Swim testing was conducted in a Plexiglas cylinder 65 cm tall, 30 cm in diameter, filled with 50 cm of water (~25° C) in a room illuminated with a red light. Swim testing occurred four hours post-infusion (post-lights off). Animals were infused and to prevent the animals from sinking or drowning "water wings" were attached. The "water wings" consist of two bubbles side by side, constructed from large "shipping-bubble-wrap" and are attached to the animal with a strip of ¼ inch adhesive tape from the midscapular area to the sternum. The animal was dropped into the swim-tank from ~20 cm above the water level.

The duration of struggling and floating, the two types of swim-test behaviors, were recorded for a total of 900 seconds (15 min.). Struggling is defined as the movement of all paws with the forepaws breaking the surface of the water. Floating is defined as a semi-motionless state with no voluntary movement of the head or limbs except when the rat "pushes off" the side of the tank to prevent from hitting the sides. After the completion of the swim test, the animal was removed from the swim-tank, dried, and returned to its home cage.

Statistical Analyses

Spontaneous Motor Activity

All analyses of motor activity were carried out on each set of animals separately. Baseline motor activity for the 0.4 ng IL-1β/rat and the 1.6 ng of IL-1β/rat and their

respective CSF groups was analyzed by a RM-ANOVA factorial design (12 [hour] X 2 [drug level]). Post-infusion motor activity for the 0.4 ng IL-1β/rat and the 1.6 ng of IL-1β/rat and their respective CSF groups was analyzed by a RM-ANOVA factorial design (12 [hour] X 2 [drug level]). Each hour of post-infusion motor activity for the 0.4 ng IL-1β/rat and the 1.6 ng of IL-1β/rat and their respective CSF groups was analyzed by a separate one-way ANOVA (1 X 2 [drug level]).

Food and Water Intake, Home-Cage Behaviors, and Swim-Test Behaviors

All analyses of these measures were carried out by a 2 X 2 ANOVA (drug [IL-1β (0.4 or 1.6 ng /rat] vs. CSF [CSF for the 0.4ng group and for the 1.6ng group] X set of animals [the 0.4 ng set or the 1.6 ng set]). These included baseline food and water intake, post-infusion food and water intake, individual home-cage behaviors, and swim-test behaviors (struggling and floating). Following the ANOVAs and where appropriate, *post hoc* analyses were conducted by a Student-Newman-Keuls test.

Results

Spontaneous Motor Activity

0.4 ng IL-1β/rat and its respective CSF group

A RM-ANOVA (hour X drug level) analyzing baseline motor of the 0.4 ng IL-1β/rat group and its respective CSF group yielded a main effect of hour [$F(11, 44) = 6.661$, $p < .001$] and a statistically significant two-way interaction [$F(11, 44) = 2.08$, $p = .042$] (Figure 13). The remaining main effect of drug level was not statistically significant. However, inspection of the data suggested that activity in hour 12, which is

Infusions of CSF, 0.4 ng or 1.6 ng of IL-1β into the LC Region
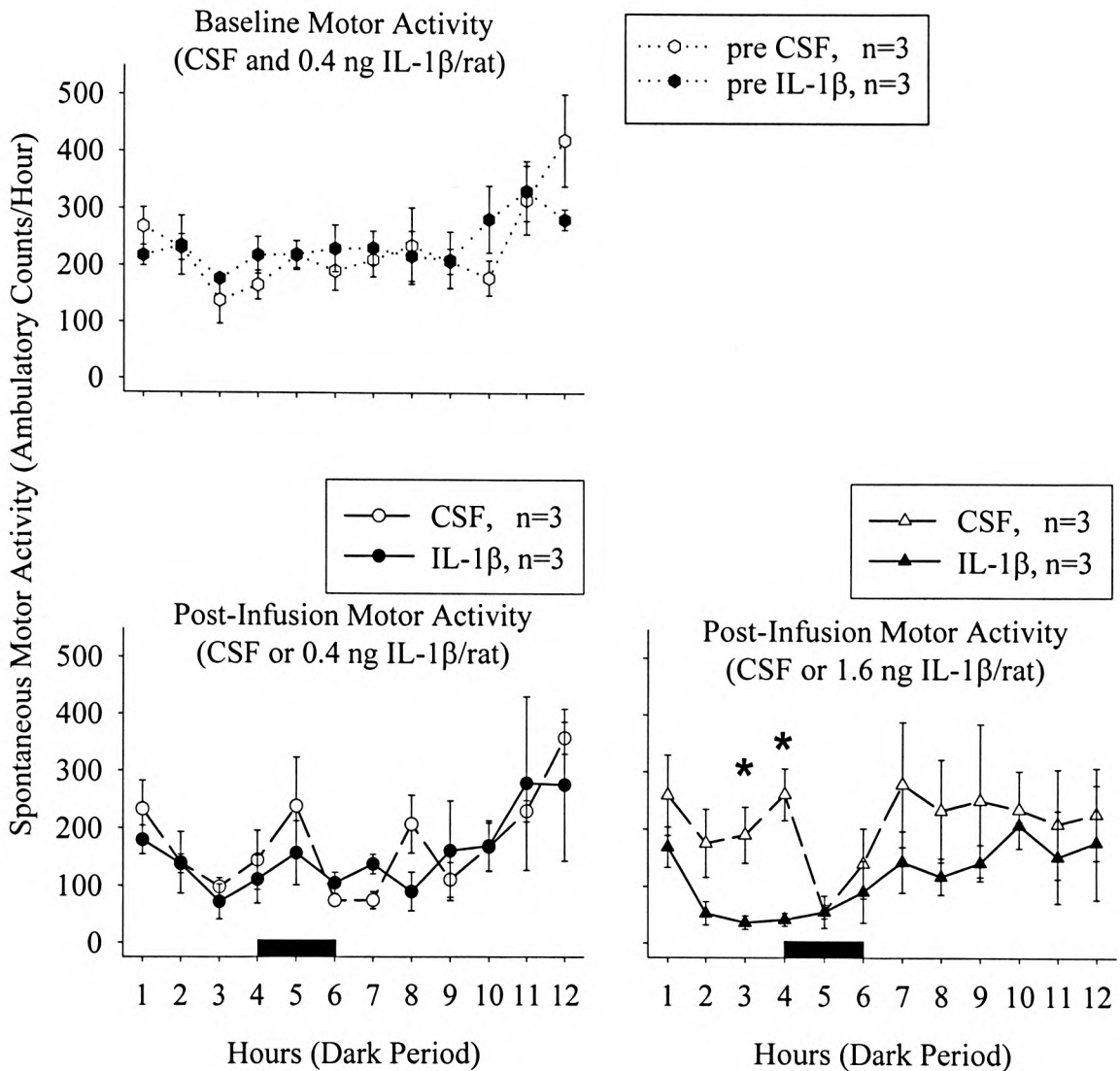
Figure 13. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats that have not been infused (baseline, top left) and of rats infused (post-infusion) with CSF or IL-1β (0.4 or 1.6 ng/rat) into the LC region (bottom left and right). The horizontal black bar along the abscissa designates the time period in which the investigator is in the room scoring the behavior of the animals and pertains only to the post-infusion data. The asterisk (*) designates a significance level of $p < 0.05$. * = 1.6 ng IL-1β/rat vs. its respective CSF group.

not affected by infused IL-1β, might be responsible for the significant interaction. When baseline motor activity was analyzed by a RM-ANOVA (hour X drug level) eliminating hour 12, the analysis yielded a main effect of hour [$\underline{F}(10, 40) = 5.48$, $\underline{p} < .001$] but the main effect of drug level and the two-way interaction did not reach statistical significance. This analysis indicated that baseline motor activity of the groups was similar.

A RM-ANOVA (hour X drug level) analyzing post-infusion motor activity yielded a main effect of hour [$\underline{F}(11, 44) = 3.84$, $\underline{p} = .001$] which was derived from a rise in motor activity toward the end of the dark period (Figure 13). The remaining main effect of drug level and the two-way interaction (hour X drug level) were not statistically significant. In separate one-way ANOVAs (each hour X drug level) of post-infusion motor activity, no effect of drug level at any hour reached statistical significance.

1.6 ng IL-1β/rat and its respective CSF group

A RM-ANOVA (hour X drug level) analyzing baseline motor of the 1.6 ng IL-1β/rat group and its respective CSF group yielded a main effect of hour [$\underline{F}(11, 44) = 2.96$, $\underline{p} = .005$]. The remaining main effect of drug level and the two-way interaction were not statistically significant. This analysis indicated that, while baseline motor activity differed depending on the hour of dark period, there was no difference in baseline motor activity at any hour between any of the groups prior to treatment.

A RM-ANOVA (hour X drug level) analyzing post-infusion motor activity did not yield any statistically significant main effects of hour, drug level, or the two-way interaction. However, the main effect of drug level approached statistical significance

[F(1, 4) = 5.85; p = .073] (Figure 13). Separate one-way ANOVAs (each hour X drug level) of post-infusion motor activity yielded a statistically significant difference at two hours (Figure 13). Specifically, 1.6 ng of IL-1β/rat produced a decrease in motor activity at hour three [F(1, 5) = 9.20, p = .039] and hour four [F(1, 5) = 22.05; p = .009] post-infusion.

Food and Water Intake

A 2 X 2 ANOVA (drug [IL-1β (0.4 or 1.6 ng /rat] vs. CSF [CSF for the 0.4ng group and for the 1.6ng group] X set of animals [the 0.4 ng set or the 1.6 ng set]) of baseline food and water intake did not yield main effects or interactions that were statistically significant. These analyses indicate that baseline food and water intake did not differ across the IL-1β-infused groups and their respective CSF-infused groups. The 2 X 2 ANOVAs of post-infusion food and water did not yield any statistically significant differences except for a main effect of set of animals in the water intake analysis [F(1, 11) = 5.98, p = .040]. The significant main effect in the water intake analysis indicates that the amount of water drank by the first set of animals (0.4 ng IL-1β/rat group and its respective CSF group) was different from (less than) the second set of animals (1.6 ng IL-1β/rat group and its respective CSF group); see Figure 14.

Home-Cage Behaviors

A 2 X 2 ANOVA (drug [IL-1β (0.4 or 1.6 ng /rat] vs. CSF [CSF for the 0.4ng group and for the 1.6ng group] X set of animals [the 0.4 ng set or the 1.6 ng set]) of individual home-cage behaviors did not yield any statistically significant differences

Infusions of CSF, 0.4 ng or 1.6 ng of IL-1β into the LC Region
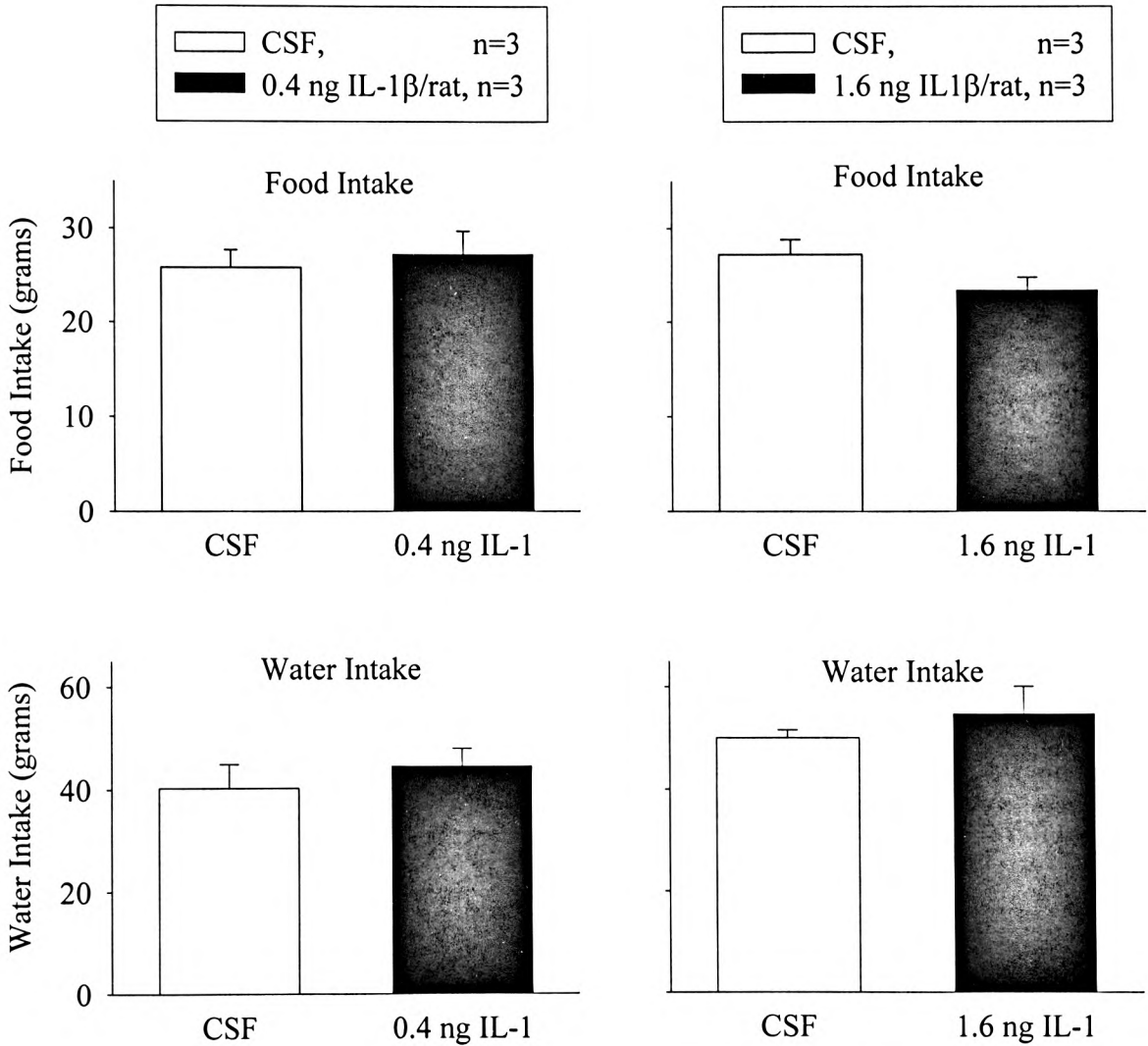
Figure 14. Mean (±SEM) food and water intake of rats infused with CSF or 0.4 ng of IL-1β/rat (top left and bottom left) and of rats infused with CSF or 1.6 ng of IL-1β/rat (top right and bottom right) into the LC region.

except for a main effect of set of animals for the curled (awake) behavior [$F(1, 11) =$ 5.41, $p = .048$]. This indicates that the time spent expressing the curled (awake) behavior by the first set of animals (0.4 ng IL-1β/rat group and its respective CSF group) was different from (more than) the second set of animals (1.6 ng IL-1β/rat group and its respective CSF group); see Figure 15.

Swim-Test Behaviors

A 2 X 2 ANOVA (drug [IL-1β (0.4 or 1.6 ng /rat] vs. CSF [CSF for the 0.4ng group and for the 1.6ng group] X set of animals [the 0.4 ng set or the 1.6 ng set]) of struggling behavior yielded a significant main effect of drug [$F(1, 12) = 7.78$, $p = .021$] (Figure 16). This indicates that animals infused with IL-1β struggled more than animals infused with CSF. The remaining main effect of set of animals and the interaction were not statistically significant. The 2 X 2 ANOVA (drug [IL-1β (0.4 or 1.6 ng /rat] vs. CSF [CSF for the 0.4ng group and for the 1.6ng group] X set of animals [the 0.4 ng set or the 1.6 ng set]) of floating behavior did not yield any statistically significant effects (Figure 16). However, inspection of the raw data showed that the above statistics were possibly affected by one score from an animal in the CSF group (the respective CSF group for the 1.6 ng IL-1β/rat group) that floated for a long period. If that single high score is omitted, the 2 X 2 ANOVA yields a significant main effect of drug [$F(1, 11)$ 6.53, $p = .034$]; see Figure 16. This indicates that animals infused with IL-1β tended to float more than animals infused with CSF.

Infusions of CSF, 0.4 ng or 1.6 ng of IL-1β into the LC Region
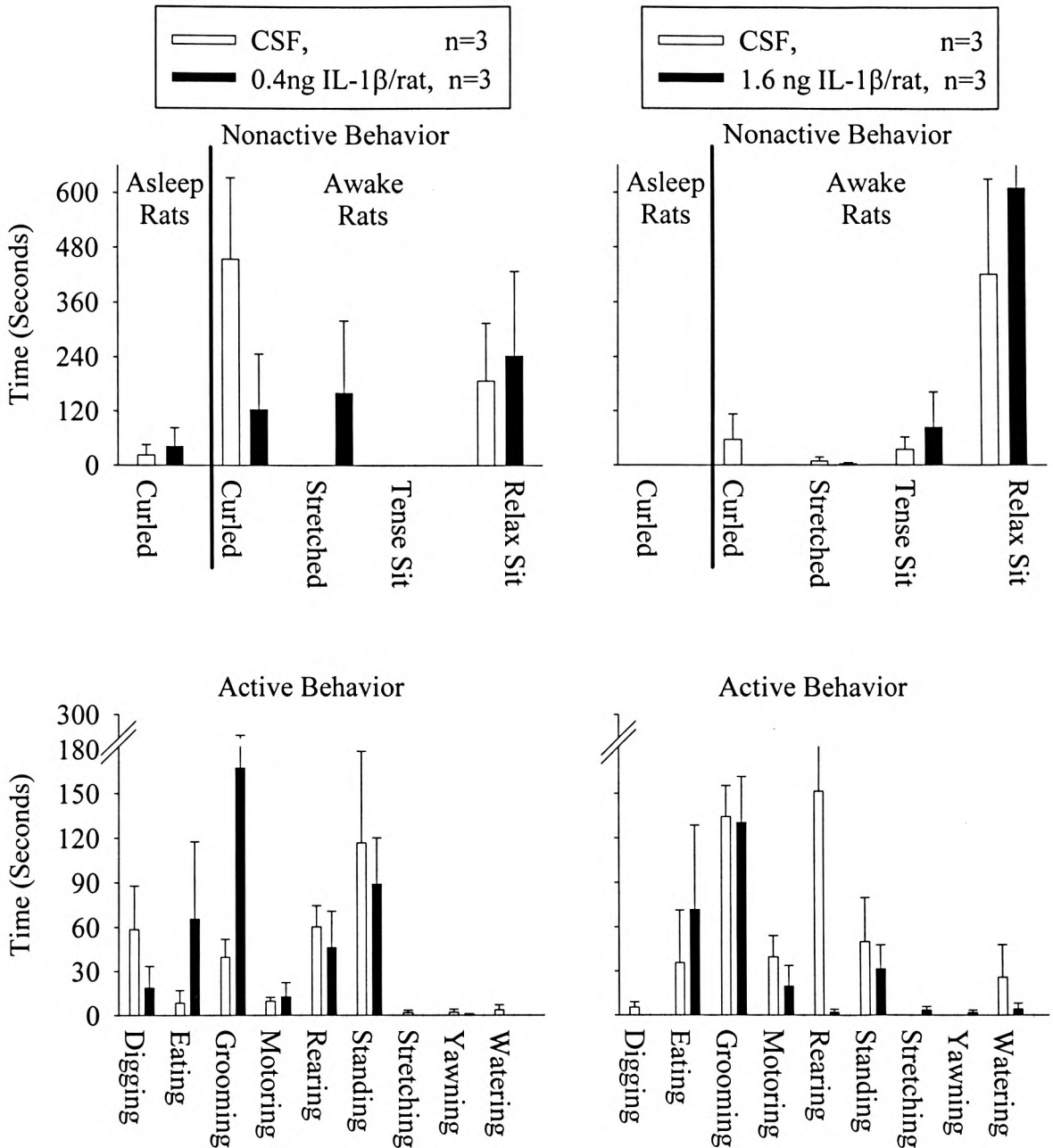
Figure 15. Mean (±SEM) nonactive and active home-cage behaviors of rats infused with CSF or 0.4 ng of IL-1β/rat (top left and bottom left) and of rats infused with CSF or 1.6 ng of IL-1β/rat (top right and bottom right) into the LC region.

Infusions of CSF, 0.4 ng or 1.6 ng of IL-1β into the LC Region
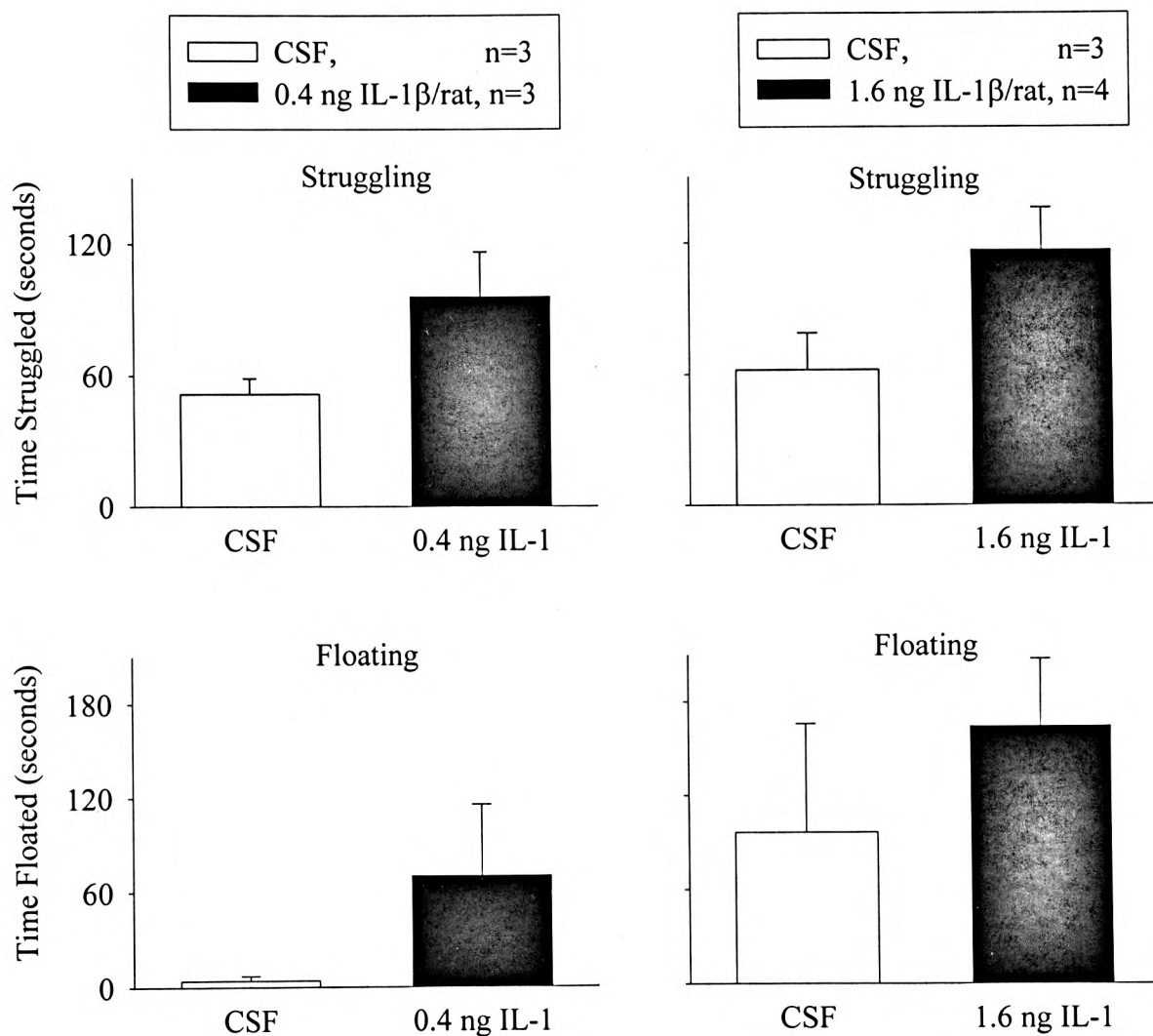
Figure 16. Mean (±SEM) struggling and floating (swim-test behaviors) of rats infused with CSF or 0.4 ng of IL-1β/rat (top left and bottom left) and of rats infused with CSF or 1.6 ng of IL-1β/rat (top right and bottom right) into the LC region.

Discussion

Spontaneous Motor Activity

Measurement of baseline motor activity for the pre-0.4 ng IL-1β/rat and its respective pre-CSF group revealed that the two groups began the experiment with similar motor activity when hour 12 is omitted from the data. Measurement of baseline motor activity for the pre-1.6 ng IL-1β/rat and its respective pre-CSF group revealed that these two groups began the experiment with comparable spontaneous motor activity.

RM-ANOVAs (hour X drug level) of post-infusion motor activity for the 0.4 ng IL-1β/rat and the 1.6 ng IL-1β/rat and their respective CSF groups both yielded a main effect of hour. However, the main effect of hour is not of particular interest; what is of interest is the main effect of drug level or the two-way interaction. In the analysis of the 1.6 ng IL-1β/rat and its respective CSF group, the effect of drug level approached significance (p = .073). Moreover, separate one-way ANOVAs (each hour X drug level) indicate that 1.6 ng IL-1β/rat significantly decreased spontaneous motor activity at hours three and four post-infusion. It can be noted that these differences were seen despite the fact that the presence of the investigator in the activity room (in order to record home-cage behaviors) apparently decreased activity of the CSF-infused group in the hours when the investigator was present. Such results indicate that 1.6 ng of IL-1β/rat was sufficient to decrease motor activity. However, similar analyses of the effects seen from an infusion of 0.4 ng IL-1β/rat yielded neither a significant main effect of drug level nor a significant two-way interaction. Also, separate one-way ANOVAs (each hour X drug

level) indicated that 0.4 ng IL-1β/rat did not decrease spontaneous motor activity at any hour.

It is unclear as to why 0.4 ng IL-1β/rat infused into the LC region did not produce a significant decrease in spontaneous motor activity as seen in Exp. 3. Perhaps the small number of animals in this experiment is the reason for the lack of significance. However, it is noteworthy that home-cage behaviors of the 0.4 ng IL-1β/rat and its respective CSF group also did not differ (Figure 15).

Food and Water Intake

Measurement of baseline food and water intake for the groups that received IL-1β (0.4 ng IL-1β/rat and the 1.6 ng IL-1β/rat) and for their two respective CSF groups revealed that the groups began the experiment with comparable intake prior to drug administration. The analyses of post-infusion food and water intake indicate that an infusion of IL-1β did not decrease food or water intake (Figure 14). However, it is possible that both doses of IL-1β (0.4 and 1.6 ng/rat) decreased food and water intake but because of the method in which these variables were measured the decreases were not detected. If IL-1β decreases food and water intake during the early portion of the dark period, and rats eat and drink throughout their active period, then they could have compensated by eating slightly more during the remaining part of the dark period. If this were true, the decrease in food and water intake would not have been detected 12+ hours after an infusion of IL-1β into the LC region.

Home-Cage Behaviors

Analysis of post-infusion nonactive and active home-cage behaviors indicated that IL-1β did not affect any home-cage behavior differently from CSF (Figure 15). Although statistical analyses indicate that the first set of animals (0.4 ng IL-1β/rat group and its respective CSF group) was different from (i.e. spent more time in the curled [awake] position) the second set of animals (1.6 ng IL-1β/rat group and its respective CSF group), it is perhaps noteworthy that within each set of animals there were differences (not statically significant) between the IL-1β-infused groups and their respective CSF-infused groups. Specifically for nonactive behavior, the 0.4 ng IL-1β/rat group spent approximately four times less in the curled (awake) position than its respective CSF group. For active behaviors, the 0.4 ng IL-1β/rat group spent approximately four times longer grooming than its respective CSF group, but the 1.6 ng IL-1β/rat group only spent a mean of 2.0 seconds rearing while its respective CSF group spent a mean of 151.67 seconds rearing. IL-1β-infused rats expressed nonactive behaviors as well as active behaviors. This suggests that IL-1β does not decrease spontaneous motor activity by immobilizing the rats or by causing lethargic behavior.

Swim-Test Behaviors

Analysis of post-infusion swim-test behavior indicate that animals infused with IL-1β struggled more and floated more than animals infused with CSF. An analysis of post-infusion struggling behavior yielded a main effect of drug. This indicates that IL-1β-infused animals struggled more than did CSF-infused animals. With respect to

floating behavior, the 2 X 2 ANOVA that included all of the animals did not yield statistically significant effects. However, the variance was evidently affected by a single high score of a CSF-infused animal. The 2 X 2 ANOVA omitting this high scoring animal yielded a significant main effect of drug. Collectively, the results from the swim test indicate that IL-1β increased both struggling and floating behavior. Although previous experiments have shown that IL-1β infused into the LC region decreases spontaneous motor activity, the fact that struggling behavior in the swim test was increased in IL-1β-infused animals suggests that the effect of IL-1β should not be characterized simply as an immobilization of the animal.

CHAPTER EIGHT

Experiment 6: Central IL-1ra and Intraperitoneal LPS

Rationale

Intraperitoneally injected LPS causes the induction of IL-1 in brain microglia. If the production of IL-1 in the brain mediates the decrease in spontaneous motor activity after ip LPS, then interleukin-1 receptor antagonist (IL-1ra) given centrally should block the effects of ip LPS. IL-1ra is a protein that is a specific antagonist for IL-1 Type I receptors. Moreover, if increased activity of LC neurons produced by IL-1 causes a decrease in motor activity after ip. LPS, then IL-1ra infused into the LC region, and not into LC surrounding regions, should prevent the decrease in motor activity after ip LPS. Experiment 6 tested whether IL-1ra infused into the LC region would block the decrease in spontaneous motor activity in response to an injection of ip LPS.

Methods

Surgical, General, Spontaneous Motor Activity, and Infusion Procedures

The procedural methods are similar to all of the previous experiments. Briefly, male rats were obtained from our group housed colony and single housed in a room where spontaneous motor activity was monitored by a computer-controlled system. After several days of housing under these conditions, animals underwent surgery. The animals were anesthetized with Halothane and implanted with cannulae aimed at the LC (n=20), lateral to the LC (l-LC), medial to the LC (m-LC), the cerebral aqueduct (AQ), the fourth ventricle (4V), and the area postrema/nucleus of the solitary tract (AP/NST); See Table 1. The latter five groups contained 6-7 animals per group. Seven to ten days after surgery,

spontaneous motor activity was recorded for three days and a mean of these three days was calculated, representing baseline motor activity for the animals. On each of these three days, the animals also were handled. Based on their baseline motor activity, the animals with a cannula aimed at the LC region were assigned to three groups. One LC group was infused with CSF and injected with ip LPS (LC-CSF + ip LPS) and another LC group was infused with IL-1ra and injected with ip phosphate buffered saline vehicle (LC-IL-1ra + ip VEH). Drug administration for the remaining LC groups will be explained below. The LC-CSF + ip LPS group was predicted to be a positive control condition since CSF infused into the LC region should have no effect and therefore motor activity should be decreased by the ip LPS. The LC-IL-1ra + ip VEH group was predicted to be a negative control condition since IL-1ra infused into the LC was also expected to have no effect and therefore motor activity should not be affected by ip VEH. The remaining LC group as well as all of the other groups whose animals had cannula aimed at other brain regions (AQ, l-LC, m-LC, 4V, and the AP/NST) were infused with IL-1ra and injected with ip LPS. The animals were infused with CSF or IL-1ra (40 μg/rat) in a total volume of 2 μl/rat, and the central infusion was immediately followed by an ip injection of VEH or LPS (10μg/kg of body weight). Infusions/injections began 1 hour prior to lights out (lights out – 19:00 hrs.). After the infusion/injection, the animals were returned to their home cage and motor activity was recorded for 12 hrs. post-infusion/injection during the dark period.

<u>Statistical Analyses</u>

Baseline motor activity for all groups was analyzed by a RM-ANOVA factorial design (12 [hour] X 8 [groups; i.e., LC-CSF + ip LPS, LC-IL-1ra + ip VEH, LC, l-LC, m-LC, AQ, 4V, and AP/NST]). Post-infusion motor activity of the positive and negative control conditions (LC-CSF + ip LPS group and the LC-IL-1ra + ip VEH group) was analyzed by a RM-ANOVA (12 [hour] X 2 [conditions]). Post-infusion motor activity of all groups was analyzed by a RM-ANOVA (12 [hour] X 8 [groups]). Finally, each hour of post-infusion motor activity was analyzed by a separate one-way ANOVA (1 X 8 [groups]). Following each of the separate one-way ANOVAs at each hour and where appropriate, *post hoc* analyses were conducted by a Dunnett's test comparing the positive control condition (LC-CSF + ip LPS) with each of the other seven groups.

<div align="center"><u>Results</u></div>

A RM-ANOVA (12 [hour] X 8 [groups]) analyzing baseline motor activity yielded a significant main effect of hour ($\underline{F}(11, 550) = 22.09$, $\underline{p} < .001$]. The remaining main effect of group and the two-way interaction were not statistically significant. This analysis indicated that, while baseline motor activity differed depending on the hour of the dark period, there was no difference in baseline motor activity at any hour between any of the groups prior to treatment.

A RM-ANOVA (12 [hour] X 2 [conditions]) analyzing post infusion motor activity of the positive and negative conditions (LC-CSF + ip LPS group and the LC-IL-1ra + ip VEH group, respectively) yielded a significant main effect of hour ($\underline{F}(11, 121) = 5.98$, $\underline{p} < .001$] which was derived from a "U" pattern of motor activity seen during the

dark period of post-infusion/injection (Figure 17, top left). The remaining main effect of condition did not reach statistical significance. However, the two-way interaction approached statistical significance [$\underline{F}$(11, 121) = 1.79, p = .063]. Inspection of the data showed that large amounts of variability occurred during hours 1, 2, 11, and 12, and these hours generally are not affected by ip LPS. Therefore, post-infusion motor activity of the positive and negative conditions were reanalyzed by a RM-ANOVA (8 [hour] X 2 [conditions]) that eliminated hours 1, 2, 11, and 12. The reanalysis no longer yielded a significant main effect of hour, but did yield a main effect of condition ($\underline{F}$(1, 11) = 5.65, $\underline{p}$ = .037]. This main effect of condition was derived from the decrease in spontaneous motor activity seen in the LC-CSF + ip LPS group when compared to the LC-IL-1ra + ip VEH group. The two-way interaction was not statistically significant.

A RM-ANOVA (12 [hour] X 8 [group]) analyzing post-infusion motor activity yielded a main effect of hour [$\underline{F}$(11, 550) = 36.64, $\underline{p}$ < .001] and was derived from a "wide-U" pattern of motor activity seen during the dark period of post-infusion/injection (Figure 17, bottom graphs). The remaining main effect of group did not reach statistical significance. However, the two-way interaction approached statistical significance [$\underline{F}$(77, 550) = 1.30, p = .053]. A significant two-way interaction indicates that groups included in this experiment, in response to the treatments administered, showed different effects on motor activity across hours of the dark period.

Since the interaction term in the previous analysis approached statistical significance, and it was important to thoroughly examine whether IL-1ra was able to block the effects of ip LPS (especially in the LC region) differentially when infused into

Central IL-1ra and Intraperitoneal LPS
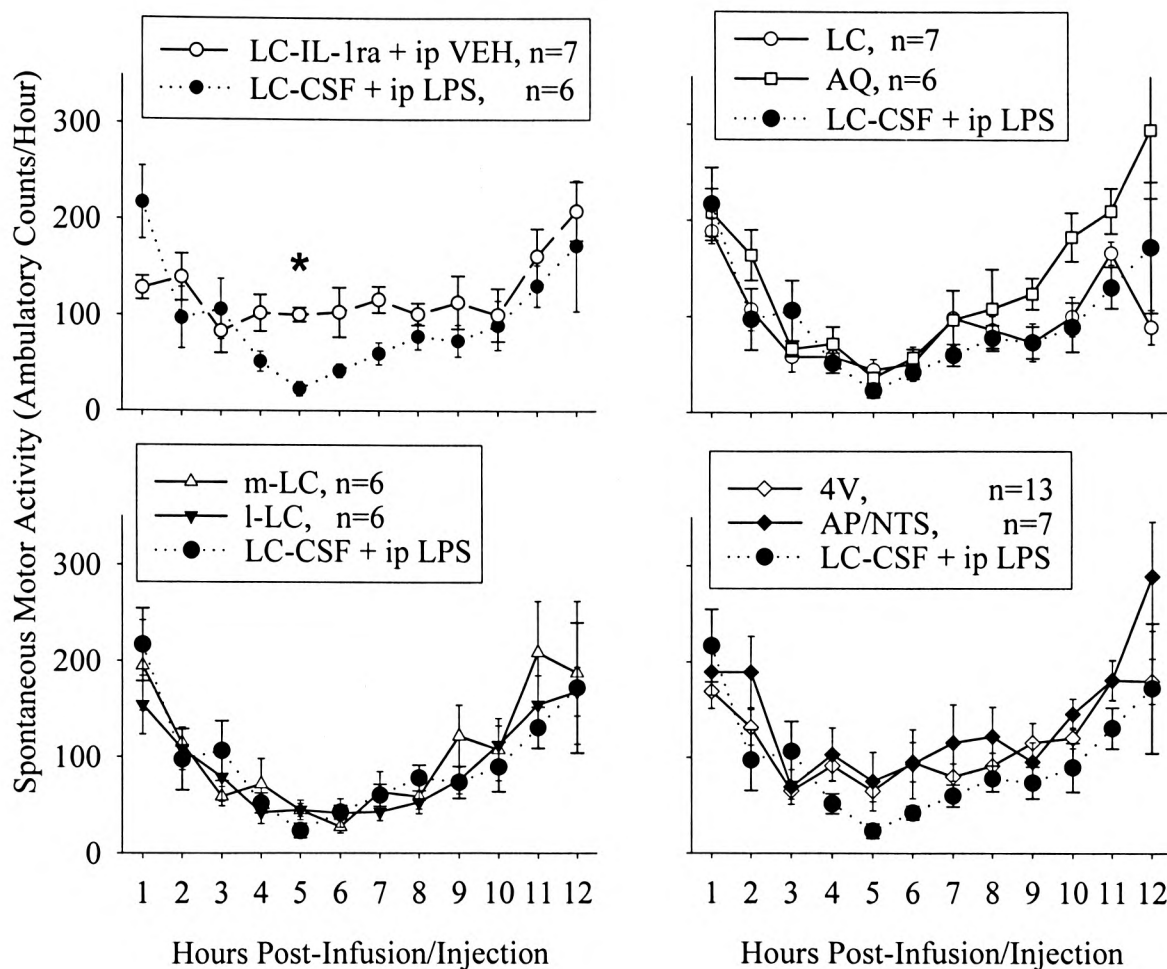
Figure 17. Mean (±SEM) ambulatory counts/hour (dark period spontaneous motor activity) of rats centrally infused with CSF or IL-1ra (40 µg/rat) into the LC or LC surrounding regions. Central infusions were immediately followed by an ip injection of VEH or LPS (10 µg/kg).

The asterisk (*) designates a significance level of $p < 0.05$ and signifies that the LC-IL-1ra + ip VEH group is different from the LC-CSF + ip LPS group. Note that the LC-CSF + ip LPS group is included in all of the graphs for easier inspection of the data.

other brain regions, another RM-ANOVA (12 [hour] X 6 [group]) of post-infusion motor activity was conducted using only the data from the six groups that were infused with IL-1ra and injected with ip LPS. This RM-ANOVA yielded a main effect of hour [$F_{(11, 429)} = 31.90$, $p < .001$], which was derived from a "wide-U" pattern of motor activity seen during the dark period of post-infusion/injection. Furthermore, the remaining main effect of group and the two-way interaction both approached statistical significance [$F_{(1, 39)} = 2.38$, $p = .056$] and [$F_{(55, 429)} = 1.28$, $p = .095$], respectively. However, inspection of the data showed that large amounts of variability occurred during hours 1, 2, 11, and 12, and these hours generally are not affected by ip LPS. Hours one and two are too early post-injection for the effects of 10 µg/kg of ip LPS, and by hours 11 and 12 post-injection the animals have already recovered from the effects of ip LPS. Therefore, post-infusion motor activity of the six groups that were infused with IL-1ra and injected with ip LPS were reanalyzed by a RM-ANOVA (8 [hour] X 6 [group]) that eliminated hour 1, 2, 11 and 12. The reanalysis by an RM-ANOVA (8 X 6) yielded a main effect of hour [$F_{(7, 273)} = 12.87$, $p < .001$]. The remaining main effect of group and the two-way interaction were not statistically significant. Collectively, the (12 [hour] X 6 [group]) and (8 [hour] X 6 [group]) RM-ANOVAs indicate that the p values found in the (12 [hour] X 6 [group]) RM-ANOVA are due to hours 1, 2, 11 and 12. These results indicate that animals infused with IL-1ra into LC and LC surrounding regions and then injected with ip LPS showed similar decreases in motor activity across the hours that are generally affected by 10 µg/kg of ip LPS (hours 3-10).

Finally, analyzing post-infusion/injection motor activity by separate one-way ANOVAs (each hour X 8 [groups]) of all eight groups yielded a significant difference at hour five [$\underline{F}$(7, 57) = 2.81, p = .015] (Figure 17). Dunnett's tests performed at hour five comparing the positive control condition (LC-CSF + ip LPS) with the seven remaining groups yielded a single statistically significant difference that was derived from a non-decrease in motor activity of the negative control group (LC-IL-1ra + ip VEH); see Figure 17.

Discussion

Measurement of baseline spontaneous motor activity revealed that the eight groups began the experiment with comparable motor activity. When baseline motor activity was analyzed by a RM-ANOVA (hour X group) the significant main effect of hour can be described by a rise in motor activity toward the end of the dark period. However, the main effect of group and the two-way interaction were not statistically significant.

The prediction that the LC-CSF + ip LPS and the LC-IL-1ra + ip VEH were positive and negative control conditions was confirmed by the findings. For the positive control condition, CSF infused into the LC region should have no effect and therefore motor activity should be decreased by the ip LPS. Figure 17 (top left) shows that ip LPS effectively decreased spontaneous motor activity for approximately four hours post-injection. These results are similar to the decreases in motor activity in response to ip LPS seen in Exp. 1. For the negative control condition, IL-1ra infused into the LC was also expected to have no effect and therefore motor activity should not be decreased by ip

VEH. Figure 17 (top left) shows that ip VEH did not decrease spontaneous motor activity, as seen in the LC-CSF + ip LPS. Comparison of the LC-CSF + ip LPS group with the LC-IL-1ra + ip VEH group showed statistically significant differences in spontaneous motor activity in the analysis of hours 3-10, which is when ip LPS affects spontaneous motor activity.

Post-infusion/injection motor activity analyzed by a RM-ANOVA (12 [hour] X 8 [group]) yielded a two-way interaction that approached statistical significance (p = .053). A two-way interaction in this experiment indicates that the groups, in response to the treatments administered, showed different effects on motor activity across hours of the dark period. Specifically, the two-way interaction could refer to motor activity of the LC-CSF + ip LPS and the LC-IL-1ra + ip VEH groups differing from one another across hours of the dark period. It could also refer to motor activity of these two conditions differing from the remaining groups that received central IL-1ra + ip LPS across hours of the dark period. Finally, it could also refer to one of the groups that received central IL-1ra + ip LPS differing from another group that also received central IL-1ra + ip LPS. An inspection of Figure 17 shows that the LC-IL-1ra + ip VEH group did not show a decrease in spontaneous motor activity as did the remaining seven groups. Therefore, it appears that the two-way interaction term refers to the LC-IL-1ra + ip VEH group differing from all other groups.

Since post-infusion/injection motor activity analyzed by a RM-ANOVA (12 [hour] X 8 [group]) yielded a two-way interaction that approached statistical significance (p = .053), and the two-way interaction term refers to several possible interactions

between the groups, it was important to determine if the interaction term referred to a significant difference between any of the six groups that were infused with IL-1ra and injected with ip LPS. All of the groups can be seen in Figure 17. As suggested by visual comparison of these groups, statistical analyses indicate that all six groups showed similar decreases in spontaneous motor activity during the middle portion of the dark period (hours 3-10).

IL-1ra infused into the LC region and LC surrounding regions did not block or attenuate the expected decrease in spontaneous motor activity in response to ip LPS. Although central IL-1ra was expected to block the decrease in motor activity that was anticipated to occur in the LC-CSF + ip LPS group, all of the groups that received ip LPS (including those that were infused with IL-1ra) showed a similar decrease in spontaneous motor activity. IL-1ra was unable to attenuate the decrease in spontaneous motor activity seen after an injection of ip LPS. IL-1ra also was unable to delay the onset of ip LPS decreases in spontaneous motor activity and unable to shorten the time to recover from a decrease in motor activity produced by ip LPS. These results indicate that the dose of IL-1ra (40 μg/rat) administered in this experiment was unable to block or affect the decrease in spontaneous motor activity seen after an injection of ip LPS (10 μg/kg).

CHAPTER NINE

Conclusions

Collectively, the experiments conducted tested whether the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region. Exp. 1 tested whether a range of ip LPS doses would cause dose-dependent decreases in spontaneous motor activity. Exp. 2, 3 and 4 tested whether LPS or IL-1β infused into the LC region or LC surrounding regions would cause a similar decrease in spontaneous motor activity as seen after ip LPS. Exp. 5 measured, in addition to changes in spontaneous motor activity, changes in food and water intake, home-cage behaviors, and swim-test behaviors in response to an infusion of IL-1β into the LC region. Finally, Exp. 6 tested whether IL-1ra infused into the LC region would block the decrease in spontaneous motor activity in response to an injection of ip LPS.

The primary purpose of Exp. 1 was to test whether a range of ip LPS doses would cause dose-dependent decreases in spontaneous motor activity. The highest doses of LPS used tended to decrease spontaneous motor activity somewhat more than the lowest dose of LPS. A secondary purpose of Exp. 1 was to test whether handling the animals, a procedure that was needed for all of the infusion experiments (Exps. 2-6), would significantly alter the overall effects in response to ip LPS. Results showed that ip LPS produced a similar decrease in spontaneous motor activity regardless of whether animals were handled or nonhandled.

The primary purpose of Exp. 2 was to test whether LPS infused into the LC region would cause a decrease in spontaneous motor activity that is similar to the

decrease seen after ip LPS. Indeed, LPS infused into the LC region decreased spontaneous motor activity in a time- and dose-dependent manner. The onset of effect for all the doses of infused LPS that significantly decreased spontaneous motor activity occurred three hours post-infusion. It is interesting that regardless of the dose of LPS, when an effect was seen, it first appeared three hours post-infusion. This three-hour delay in the onset of the decrease in motor activity may be explained by the time required for LPS to cause the induction of IL-1 in the brain. But while effective doses showed initial effects three hours post-infusion, the highest doses of LPS then suppressed spontaneous motor activity more than did the lowest dose of LPS when activity decreases appeared. Moreover, the time to recover from the effects of LPS was more delayed in the groups that received the highest doses of LPS than in the group that received the lowest doses, suggesting that the highest doses of LPS maintain the induction of IL-1 for longer periods than the lowest doses of LPS.

The primary purpose of Exp. 3 was to test whether IL-1 directly infused into the LC region would cause a decrease in spontaneous motor activity that is similar to the decrease seen after ip LPS (Exp. 1) or an infusion of LPS into the LC region (Exp. 2), thereby further suggesting that ip LPS or LPS infused into the LC region acts by stimulating IL-1 in the LC region. Indeed, a dose-dependent decrease in spontaneous motor activity was seen when IL-1β was infused into the LC region, and the decrease was similar to what was seen after ip LPS or after an infusion of LPS into the LC region. Similarities of the effects produced by ip LPS, LPS into the LC region, or IL-1β into the LC region were as follows: 1) all three treatments produced a decrease in motor activity

that was roughly similar in magnitude and pattern, 2) the onset of the decrease in motor activity occurred three hours after a drug was administered, and 3) by the end of the dark period, all treatments groups had recovered from the decrease in spontaneous motor activity.

Questions arise from the observation that the onset of the decrease in motor activity occurred at the same time in animals that received ip LPS, LPS into the LC region, or IL-1β into the LC region. Previous findings have shown that LPS causes the induction of IL-1 in the brain (Quan et al., 1994; Weiss et al., 1994). If ip LPS (Exp. 1) or infusions of LPS into the LC region (Exp. 2) produce a decrease in motor activity via induction of IL-1 in the brain to stimulate LC neurons, then an infusion of IL-1β directly into the LC region should decrease spontaneous motor activity sooner than LPS (peripheral or central). Whereas LPS must first cause the induction of IL-1 to bring about the decrease in spontaneous motor activity, IL-1 directly infused into the LC region should eliminate the need for induction of IL-1 and consequently the effects on spontaneous motor activity would be expected to occur sooner than when LPS is given. The observation that the onset of the decrease in motor activity occurred at the same time in animals that received ip LPS, LPS into the LC region, or IL-1β into the LC region suggests that both LPS and IL-1 cause the induction of some other transmitter/substance in the brain that brings about the decrease in spontaneous motor activity.

Another important point can be made regarding the similarity of spontaneous motor activity of the animals injected with ip LPS and animals infused with IL-1β into the LC region. Although as pointed out above findings from Exp. 1 and 3 reveal that rats

injected with 10 μg/kg of ip LPS showed similar decreases in spontaneous motor activity as did the rats infused with IL-1β into the LC region, a pilot study suggests that these two groups of rats are affected differently by these treatments. Animals injected with ip LPS were observed to be sedated and lethargic (pilot study) whereas such sedation was not evident when IL-1β was infused into the LC region (Exp. 5). Thus, the post-treatment behavior of these two groups of animals differs considerably despite the fact that the decrease in spontaneous motor activity recorded by the computer-controlled system was similar in both groups.

The purpose of Exp. 4 was to test whether the decrease in spontaneous motor activity seen after an infusion of IL-1β into the LC region was specific to the LC. This was done by assessing whether the decrease in motor activity would occur only if IL-1β were infused into the LC region and would not occur if IL-1β were infused into regions surrounding the LC. Unexpectedly, motor activity was similarly affected when IL-1β was infused into all brain regions including the LC region with all groups showing a moderate decrease in motor activity at hours 2-5 post-infusion. To assess whether motor activity was indeed decreased by an infusion of IL-1β, a RM-ANOVA was conducted with an added control group that showed normal activity (i.e., no decrease). The analysis indicated that an infusion of IL-1β did not produce a statistically significant decrease in spontaneous motor activity. Based on the results that had been obtained in the third experiment, 0.4 ng of IL-1β/rat had been chosen for use in Exp. 4 because this was found to be the lowest dose of IL-1β that caused a statistically significant decrease in motor

activity. It is not evident why 0.4 ng of IL-1β/rat did not yield statistically significant decreases in motor activity in Exp. 4, at least when infused into the LC region. Although in Exp. 4 the pattern of motor activity was similar when IL-1β was infused into all brain regions, comparison to a control group revealed that IL-1β did not significantly decrease motor activity when infused into any brain region including the LC. It was expected that an infusion of IL-1β into the LC region would decrease motor activity, and since this did not occur, the results from Exp. 4 were inconclusive.

The purpose of Exp. 5 was to measure changes in food and water intake, home-cage behaviors, and swim-test behaviors in addition to spontaneous motor activity in response to IL-1β infused into the LC region. Collectively, the results from Exp. 5 indicate that while such infusion decreases spontaneous motor activity, the effects from IL-1β infused into the LC region cannot be characterized as immobilizing the animal. This conclusion is supported by the observation that many normal home-cage behaviors of the IL-1β-infused animals and CSF-infused animals did not differ. Furthermore, the fact that in the swim test animals infused with IL-1β struggled more than animals infused with CSF indicates that animals infused with IL-1β can be more active than animals infused with CSF, and that animals are not immobilized or sedated by an infusion of IL-1β into the LC region.

It is interesting that although infusions of IL-1β decreased spontaneous motor activity, such infusion increased struggling behavior in the swim test. The different effects caused by IL-1β raise the possibility that spontaneous motor activity and

struggling in the swim test are different types of active behaviors. Specifically, spontaneous motor activity is measured in the home cage of an animal that undergoes little or no stress during the measurement procedure; in contrast, the swim test requires the animal to swim in a tank of cool water for 15 minutes (moderate stress). The difference in stress level between these two procedures could be responsible for the different effects of IL-1β on activity (spontaneous motor activity or activity in the swim test).

The purpose of Exp. 6 was to test whether IL-1ra infused into the LC region would block the decrease in spontaneous motor activity in response to an injection of ip LPS. IL-1ra was unable to attenuate the decrease in spontaneous motor activity seen after an injection of ip LPS. This experiment showed that IL-1ra also was unable to delay the onset of the decrease in spontaneous motor activity produced by ip LPS and was unable to shorten the time to recover from the decrease in motor activity caused by ip LPS. At least for the dose of IL-1ra (40 μg/rat) administered in this experiment, the results indicate that IL-1ra was unable to block or affect the decrease in spontaneous motor activity seen after an injection of ip LPS (10 μg/kg).

In summary, the findings do not support the hypothesis that the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region. First, IL-1ra was unable to block the decrease in spontaneous motor activity seen after an injection of ip LPS. Other findings suggest why it was that IL-1ra was unable to block the effects of ip LPS. The pilot study alluded to earlier indicated that although rats injected with 10 μg/kg of ip LPS showed a decrease in

spontaneous motor activity that was similar to what was seen in rats infused with IL-1β into the LC region (Exp. 3 and 5), the rats given ip LPS were observed to be sedated and lethargic. In contrast, the animals infused with IL-1β into the LC region (Exp. 5) were not sedated and lethargic. Thus, the post-treatment behavior of these two groups of animals differs considerably despite the fact that the decrease in spontaneous motor activity recorded by the computer-controlled system was similar in both groups. It seems likely that the decrease in spontaneous motor activity produced by ip LPS is but one aspect of a number of behavioral changes that are likely to depend on activation of many brain regions including the LC region by ip LPS, suggesting that the effects of ip LPS are more generalized than the effects of IL-1β infused into the LC region. If the effects of ip LPS are more generalized, then it is not surprising that the effects of ip LPS could not be blocked by an infusion of IL-1ra into a single brain region.

Second, it was expected that if ip LPS (Exp. 1) or infusions of LPS into the LC region (Exp. 2) produce a decrease in motor activity via the induction of IL-1 in the brain to stimulate LC neurons, then an infusion of IL-1β directly into the LC region should cause the onset of the decrease in spontaneous motor activity to occur sooner than when LPS (peripheral or central) is given. However, results showed that the onset of the decrease in motor activity for all three treatment groups occurred three hours after drug administration. That the delay of onset of effect was similar for all three treatment groups is consistent with the possibility that induction of some other transmitter/substance in the brain, which IL-1 and LPS both induce, is what brings about the decrease in spontaneous motor activity. If the effects of ip LPS are mediated by some

other substance in the brain that is not IL-1, then an infusion of IL-1ra into the brain (any region) would not be expected to block the decrease in motor activity seen after ip LPS.

In conclusion, we hypothesized that the decrease in spontaneous motor activity that occurs after an injection of ip LPS is mediated by the induction of IL-1 in the LC region. Collectively, the findings do not support the above hypothesis.

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NOTES

[1]Coronal view of the structures that were targeted with cannulae.

[2]Coronal view of cannulae that were implanted and aimed at the LC region.

[3]Coronal view of cannulae that were dorsal to the cannulae aimed at m-LC.

[4]Coronal view of cannulae that were implanted and aimed at the LC region.

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APPENDICES

Appendix A: Cannula and Infusion Needle Construction

Each cannula was constructed from 26 gauge (GA) intradermal bevel needles (Becton Dickinson), 3/8 in. in length with the plastic hub melted off (Fig. 18.1). The distal end of the needle was attached to a narrow silicone tubing [0.012 inches (in.) inter-diameter (I.D.) X 0.025 in. outer-diameter (O.D.) from Technical Products, Inc.]. The length of the narrow silicone tubing is 5 cm (Fig. 18.2). Also attached to the distal end of the needle and surrounding the narrow tubing, was a larger silicone tubing (0.040 in. I.D. X 0.085 in. O.D. from Technical Products, Inc.). The length of the larger silicone tubing is exactly 3.2 cm (Fig. 18.3). The total length of the cannula from the tip of the needle to the end of the large tubing was exactly 4.2 cm (Fig. 18.4) and to the end of the narrow tubing it is 6 cm (Fig. 18.5), so that the narrow tubing extends 1.8 cm from the end of the large tubing. To prevent clogging of the cannula after it has been implanted, an obturator (a thin piece of nylon wire) (Fig. 18.6) that is the entire length of the cannula (6 cm), is inserted into the narrow tubing and glued/sealed with silicone glue.

At the time of infusion, a stainless steel infusion needle attached to a microsyringe was inserted into the narrow silicone tubing of the implanted cannula. The infusion needle (no bevel) was constructed from 30 GA stainless steel tubing that is 4 cm in length (Fig. 18.7) surrounded by 23 GA stainless steel tubing that is 1.5 cm long (Fig. 18.8). One end of the 23 GA tubing is rounded (Fig. 18.9) and the remaining end was soldered to the 30 GA tubing (Fig. X-10). The 2 pieces of tubing, 23 GA and 30 GA, were positioned so that one end of the 30 GA tubing (the infusion needle tip) protruded exactly

1.5 cm from the rounded end of the 23 GA tubing (Fig. 18.11). The distal end of the infusion needle tip was attached to a ~75 cm polyethylene (PE 10) tubing (Fig. 18.12). The remaining end of the PE 10 tubing was attached to one end of a 3 cm PE 20 piece of tubing (Fig. 18.13) using a modified 30 GA needle as a juncture (plastic hub is melted off the 30 G needle) (Fig. 18.14). Just prior to the infusion procedure, the 75 cm tubing was backfilled (from the PE 20 tubing to the infusion needle tip) with distilled water. The PE 20 tubing was attached to a microsyringe driver [Fig. 18.15 (Delsaga microdoser)] which holds two 10μl Hamilton syringes (Fig. 18.16) that also have been previously filled with distilled water. The drug was loaded into the tip of the infusion needles and the infusion needles were inserted into the implanted cannula.
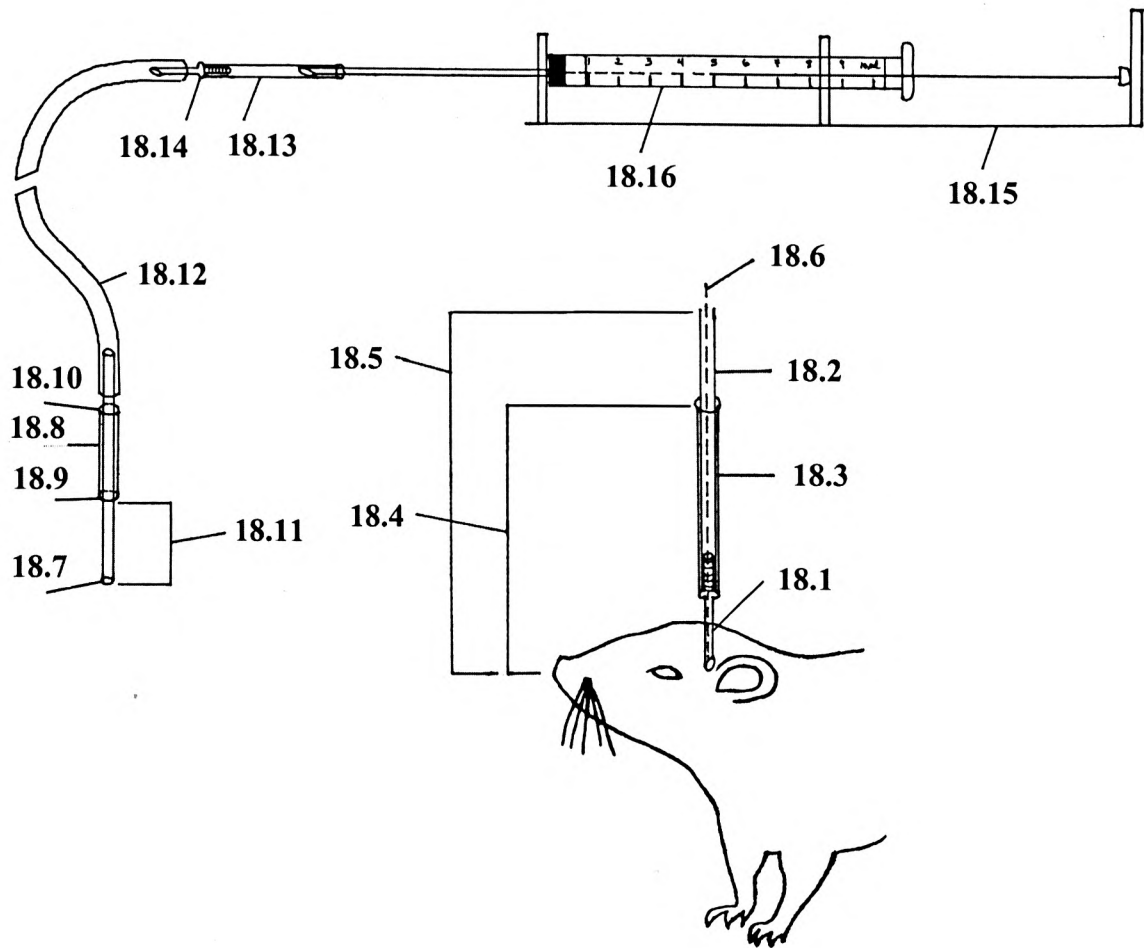
18.14   18.13

18.16

18.15

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18.10
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18.2

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18.4

18.1

Figure 18. Implanted cannula, infusion needle and microsyringe driver, in detail.

Appendix B: Home-Cage Behaviors

Behavior	Code	Description
**State of the Rat**		
Asleep	6	Any position with eyes completely closed.
Awake	7	Any position with eyes partially or totally open.
**Nonactive Behaviors of the Rat**		
Curled	1	Lying or sitting with head bent over so spine is curved.
Curled (buried)	(1)	Same as "curled" but with head buried under the body.
Stretched	2	Lying with belly touching the floor without curvature in the spine.
Tense sitting	3	Sitting with belly not touching the floor and front legs extended.
Relaxed sitting	4	Sitting with belly touching the floor, front legs tucked under body.
Motor Behavior	8	Change of position, designation given while eyes are closed (asleep).
**Active Behaviors of the Rat**		
Digging	D	Head or front paws moving corn-cob.
Eating	E	Smelling or eating food.
Grooming	G	Licking coat or paws, scratching, facial grooming with paws.
Jumping	J	All four legs not touching cage floor.
Motor Behavior	M	Walking or running.
Standing	S	Belly not touching the floor and all four paws on the floor.
Rearing	R	Body raised with front paws off cage floor.
Stretching	ST	Extension and stretching of body. Often accompanied by a yawn.
Startle	S!	Sudden and quick movement of body. Often accompanied by a small vocalization.
Watering	W	Smelling or drinking from the water bottle.
Yawing	Y	Stretching mouth by opening and closing slowly.

Table 2. Nonactive and active home-cage behaviors, codes and descriptions.
Nonactive behaviors are always accompanied by an asleep or awake score.
Active behaviors are always accompanied by an awake score.

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Attached are copies of the letters from Academic Press granting express permission to modify and print the section reproductions of the stereotaxic atlas that are included in this dissertation.

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