ANALYSIS OF BEHAVIORAL AND NEURONAL ACTIVATION FOLLOWING AMPA AND NMDA MICROINJECTIONS INTO THE PERIFORNICAL LATERAL HYPOTHALAMIC AREA IN RATS

by

Frederick Wai-Tsin Li

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

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DALHOUSIE UNIVERSITY

DEPARTMENT OF ANATOMY & NEUROBIOLOGY

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Dated: January 28, 2011

Supervisor: _____

Readers: _____

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AUTHOR: Frederick Wai-Tsin Li

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DEDICATION PAGE

To my dear parents, Cecilia & Philip Li, whose unwavering love and support have always been a pillar of strength for me.
Thank you for teaching me to never give up, to leave no job unfinished, and to always look for the positive aspect of any hardship.

TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES ix

ABSTRACT x

LIST OF ABBREVIATIONS USED xi

ACKOWLEDGEMENTS xii

CHAPTER 1: INTRODUCTION **1**

1.1. Discovery of the Hypothalamus as a Region for the Regulation of Behavioral Arousal 1

1.2. Regulation of Wake-Related Behaviors By the Posterior Hypothalamus 1

1.3. The PeFLH: A Center for the Regulation of Arousal Related Behaviors 2

1.4. Study Objectives 3

CHAPTER 2: BEHAVIORAL AND NEURONAL ACTIVATION FOLLOWING MICROINJECTIONS OF AMPA AND NMDA INTO THE PERIFORNICAL LATERAL HYPOTHALAMUS IN RATS **4**

2.1. Introduction 4

2.2. Materials and Methods 7

2.2.1. Animals 7

2.2.2. Surgery 7

2.2.3. Drugs and microinjection procedures 8

2.2.4. Behavioral experiments 9

2.2.4.1. The AMPA experiment 9

2.2.4.2. The NMDA experiment 10

2.2.5. Perfusion and tissue preparation 10

2.2.6. Immunohistochemistry 10

2.2.7. Behavioral analyses 12

2.2.7.1. Exploratory behaviors 12

2.2.7.2. Non-Exploratory behaviors 13

2.2.8. Histological Analyses 13

2.2.8.1. c-Fos immunoreactivity at the injection site 13

2.2.8.2. Orexin- and c-Fos-immunoreactive neurons in the PeFLH 14

2.2.9. Statistical Analyses 15

2.3. Results 15

2.3.1. Injection Sites 15

2.3.2. Behavioral Responses 18

2.3.2.1. AMPA Injections 18

2.3.2.1.1. Locomotor activity 18

2.3.2.1.2. Rearing 23

2.3.2.1.3. Stillness 23

2.3.2.1.4. Head Movement and Grooming 23

2.3.2.1.5. Feeding and Drinking 26

2.3.2.2. NMDA Injections 26

2.3.2.2.1. Locomotor activity 26

2.3.2.2.2. Rearing and Stillness 31

2.3.2.2.3. Head Movement and Grooming 31

2.3.2.2.4. Feeding and Drinking 31

2.3.3. c-Fos Immunoreactivity 31

2.3.3.1. Areas Surrounding the Injection Site 31

2.3.3.2. c-Fos+/OX+ Neurons in the PeFLH 32

2.3.3.2.1. AMPA Injections 39

2.3.3.2.2. NMDA Injections 39

2.3.4. Correlations Between c-Fos+/OX+ Labeling and Behavioral Measures 40

2.4. Discussion 40

2.4.1. Technical Considerations 43

2.4.2. Behavioral Responses to AMPA and NMDA Microinjections 44

2.4.3. Role of OX Neurons in Behavioral Responses Elicited by AMPA and NMDA 46

2.4.4. Conclusions 49

CHAPTER 3: DISCUSSION **50**

3.1. Future Studies 50

3.1.1. Approaches For Identifying Glutamatergic Inputs to the PeFLH 50

3.1.2. Approaches for Selective Activation of Neuronal Populations Within the PeFLH 51

3.1.3. Refining Our View of PeFLH Neuronal Populations 52

3.2. Concluding Remarks 54

REFERENCES **55**

LIST OF TABLES

Table 1	Total durations of time spent in Grooming or in Head Movement during the 45-min observation period after administration of ACSF, AMPA or NMDA	24
Table 2	Percentage of OX+/Fos+ cells and the total number of OX+ cells on the side ipsilateral or contralateral to the last microinjection in each rat	37

LIST OF FIGURES

Figure 1	Photomicrographs of an injection site within the PeFLH	16
Figure 2	Schematic drawing showing the locations of injection sites relative to the distribution of OX neurons	19
Figure 3	Distance Traveled, and times spent in Locomotion, Rearing, and Stillness following administration of ACSF, 1 mM AMPA, or 2 mM AMPA	21
Figure 4	Amounts of food consumed and time spent Drinking following microinjections of AMPA and NMDA	27
Figure 5	Distance Traveled, and times spent in Locomotion, Rearing, and Stillness following administration of ACSF, 1 mM NMDA, or 10 mM NMDA	29
Figure 6	Estimated extents of areas that were affected by ACSF, AMPA, and NMDA injections, as revealed by c-Fos immunoreactivity	33
Figure 7	Micrograph of a section double immunostained for c-Fos and OX	35
Figure 8	Correlations between OX+/Fos+ cell counts and time spent in Locomotion, Distance Traveled, time spent Rearing, and time spent in Drinking after microinjections of ACSF, AMPA, and NMDA	41

ABSTRACT

Although the perifornical lateral hypothalamic area (PeFLH), which contains orexin/hypocretin (OX) neurons, plays an important role in arousal-related behaviors, its neuromodulatory inputs are incompletely understood. The present study examined the role of glutamatergic inputs to the PeFLH in various arousal-related behaviors. Adult male rats received a microinjection of the ionotropic glutamate receptor agonists AMPA (1 and 2 mM) or NMDA (1 and 10 mM), or vehicle into the PeFLH, and were placed in an open field; 90 min later, rats were perfused for immunohistochemistry for OX and c-Fos as a marker of neuronal activation. AMPA injections dose-dependently increased locomotion, rearing, and drinking. NMDA injections (at 10 mM) increased locomotion and feeding. All these behaviors (except feeding) were positively correlated with the number of c-Fos/OX-immunoreactive neurons. These results support the role of ionotropic glutamate receptors on OX (and other) neurons in the PeFLH in the regulation of locomotor and ingestive behaviors.

LIST OF ABBREVIATIONS USED

ABC	avidin-biotin-horseradish peroxidase complex
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
DAB	diaminobenzidine
DMH	dorsomedial hypothalamic nucleus
GABA	γ-aminobutyric acid
ICV	intracerebroventricular
LC	locus coeruleus
LH	lateral hypothalamic area
LMA	locomotor activity
MCH	melanin-concentrating hormone
NMDA	N-methyl-D-aspartate
OX	orexin/hypocretin
PBS	phosphate-buffered saline
PeFLH	perifornical lateral hypothalamic area
TMN	tuberomammillary nucleus
VMH	ventromedial hypothalamic nucleus

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CHAPTER 1: INTRODUCTION

1.1. Discovery of the Hypothalamus as a Region for the Regulation of Behavioral Arousal

In the early part of the 20th century, it was recognized that the regulation of behavioral states could be localized to areas or nuclei within the hypothalamus. Some of the first evidence was provided in the early 1930s by a Viennese neurologist, Baron Constantin von Economo, who noted that patients who had prolonged states of sleepiness as a result of a viral infection in the brain had lesions to the posterior hypothalamus and the rostral midbrain (von Economo, 1930). Conversely, those that were infected but had the opposite symptom, prolonged states of insomnia, had lesions more anteriorly, in the basal forebrain and,the preoptic area of the anterior hypothalamus (von Economo, 1930). These observations led von Economo to hypothesize that neurons in the anterior hypothalamus near the optic chiasm promote sleep whereas neurons in the posterior hypothalamus promote wakefulness.

1.2. Regulation of Wake-Related Behaviors By the Posterior Hypothalamus

In the decades to follow, studies using electrical or chemical stimulation or lesion of the posterior hypothalamus in various animal species (Nauta, 1946; Swett and Hobson, 1968) further supported von Economo's hypothesis that this region is involved in the promotion of waking (Shoham and Teitelbaum, 1982; Lin et al., 1989). Further studies have revealed the presence of various loci within the posterior hypothalamus for eliciting different behaviors associated with arousal, such as feeding and drinking (Teitelbaum and Epstein, 1962; Stricker et al., 1978; Clark et al., 1991), locomotor activity (Parker and Sinnamon, 1983; Marciello and Sinnamon, 1990), as well as cardiovascular responses

(Stock et al., 1981; Allen and Cechetto, 1992) and reproductive behavior (Christensen et al., 1977; Nance et al., 1977). One area that was discovered to be involved with many of these behaviors is the perifornical lateral hypothalamic area (PeFLH).

1.3. The PeFLH: A Center for the Regulation of Arousal Related Behaviors

The PeFLH is located in the posterior hypothalamus surrounding the dorsal aspect of the fornix and, in the rat, spans approximately 1 mm longitudinally from 2.8 – 3.8 mm posterior to bregma (Palkovits, 1975; Swanson, 1987; Paxinos and Watson, 1997). Anatomically, this area is bordered medially by the dorsomedial nucleus of the hypothalamus (DMH), laterally by the internal capsule, dorsally by the subincertal nucleus and zona incerta, and ventrally by the dorsal aspect of the fornix. Stimulation of the PeFLH in rats has been shown to elicit feeding (Stanley and Thomas, 1993), drinking (Greer, 1955; Mogenson et al., 1971), and locomotor activity (Parker and Sinnamon, 1983), along with promotion of wakefulness (Koyama et al., 2003; Alam and Mallick, 2008). Furthermore, anatomical tracing studies have shown the PeFLH (Hahn and Swanson, 2010) to send efferent projections to, and to receive afferent inputs from, both adjacent and distant nuclei also known to be involved with these behaviors, including the DMH (Chou et al., 2003), ventromedial nucleus of the hypothalamus (Aravich and Beltt, 1982), subfornical organ (Miselis, 1981), and locus coeruleus (Aston-Jones et al., 1995).

Although extensive work has thus been conducted to identify the functional role of the PeFLH, it was not until the last 20 – 30 years when it became possible to identify neurotransmitter phenotypes of neurons that populate this region using immunocytochemistry. The PeFLH houses a heterogeneous population of neurons including those containing glutamate (Li et al., 2002; Collin et al., 2003), dynorphin

(Zamir et al., 1983; Li and van den Pol, 2006), melanin concentrating hormone (MCH, (Bittencourt et al., 1992)), orexin/hypocretin (OX, (de Lecea et al., 1998)), and probably other transmitters.

1.4. Study Objectives

The identification of various neuronal phenotypes within the PeFLH has provided an opportunity to take a closer look at afferent inputs to specific neuronal populations in this region and how these populations play a role in various arousal related behaviors associated with the PeFLH. The goal of the present study was to investigate the role of glutamate in the PeFLH in eliciting arousal related activities such as locomotor activity and ingestive behaviors by using ionotropic glutamate receptor agonists, AMPA and NMDA, to stimulate neurons within this region. In addition, to begin to determine the role of OX neurons in these behavioral effects, these behavioral responses were correlated to the activity of OX neurons using c-Fos immunoreactivity. This work represents an initial attempt to demonstrate how selective neuronal populations within the PeFLH are involved with arousal behaviors.

CHAPTER 2: BEHAVIORAL AND NEURONAL ACTIVATION FOLLOWING MICROINJECTIONS OF AMPA AND NMDA INTO THE PERIFORNICAL LATERAL HYPOTHALAMUS IN RATS

2.1. Introduction

The perifornical lateral hypothalamic area (PeFLH) has been implicated in a variety of behavioral and physiological functions, including feeding (Teitelbaum and Epstein, 1962; Clark et al., 1991; Stanley and Thomas, 1993), locomotion (Parker and Sinnamon, 1983; Sinnamon, 1990), cardiovascular regulation (Stock et al., 1981), and sleep and wakefulness (Shoham and Teitelbaum, 1982; Alam et al., 2002; Lu et al., 2007). The PeFLH houses a heterogeneous population of neurons including those containing glutamate (Li et al., 2002), γ-aminobutyric acid (GABA; (Abrahamson and Moore, 2001)), melanin-concentrating hormone (MCH; (Bittencourt et al., 1992; Broberger et al., 1998)), and orexin/hypocretin (OX; (de Lecea et al., 1998; Sakurai et al., 1998)). Of these cell types, the OX cell population has been the most intensely studied in the PeFLH.

OX neurons are located exclusively in the PeFLH and project throughout the CNS including sleep/wake-regulatory regions (Peyron et al., 1998; Nambu et al., 1999). Several lines of evidence implicate the major role of these neurons in arousal and motor activity. OX neurons discharge maximally during active wakefulness and are quiescent during sleep (Estabrooke et al., 2001; Lee et al., 2005; Mileykovskiy et al., 2005). CNS administration of OX increases behavioral arousal (Hagan et al., 1999; Espana et al., 2003) while the absence of OX or its receptors is associated with the sleep disorder narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Thannickal et al., 2000). OX neurons have also been proposed to play a role in food intake. Intracerebroventricular (ICV)

administration of this neuropeptide increases food intake (Sakurai et al., 1998; Hagan et al., 1999) and food anticipatory activity is reduced in OX knockout mice (Akiyama et al., 2004).

Modulation of the activity of OX neurons by their afferents has been studied in some detail. Anatomically, the possible sources of afferents specifically to OX neurons include the lateral septum, bed nucleus of the stria terminalis, several hypothalamic areas including the preoptic area, dorsomedial nucleus, and lateral hypothalamus, and brain stem regions including the periaqueductal gray matter, dorsal raphe nucleus, and lateral parabrachial nucleus (Yoshida et al., 2006). These findings largely corroborate findings of the afferent connections of the PeFLH in general (Hahn and Swanson, 2010), which originate in both telencephalic (Swanson and Cowan, 1975) and brainstem nuclei (Aston-Jones et al., 1995), as well as other hypothalamic nuclei (Chou et al., 2003). The role of these projections in modulating the activity of specific neurons within the PeFLH and physiological or behavioral consequence of such activation is not fully understood. The neurotransmitter types of many of these inputs also remain to be identified, although at least some are likely glutamatergic (Chou et al., 2003; Niu et al., 2010).

Evidence supports the role of glutamate as an important excitatory neurotransmitter in activating OX and, likely, other neurons in the PeFLH to elicit specific behaviors. Microinjections of glutamate or agonists for specific ionotropic glutamate receptor subtypes into the LH increased feeding (Stanley et al., 1993a; Stanley et al., 1996; Duva et al., 2002) and locomotor behaviors (Milner and Mogenson, 1988; Marciello and Sinnamon, 1990). Injections of glutamate into the PeFLH also promoted wakefulness while inhibiting sleep (Alam and Mallick, 2008). Consistent with these

findings, moderate to high levels of ionotropic glutamate receptor genes and proteins (Meeker et al., 1994; Khan et al., 1999; Eyigor et al., 2001), and high levels of vesicular glutamate transporter 2 immunoreactivity (Collin et al., 2003; Henny and Jones, 2006) have been reported in the PeFLH, suggesting the abundance of glutamatergic synapses. Furthermore, co-localization of glutamate and OX has been found in some OX cells (Rosin et al., 2003; Torrealba et al., 2003) and OX cells have been known to communicate with other OX cells directly (Yamanaka et al., 2010) or via a glutamate interneuron (Li et al., 2002). Despite these behavioral, anatomical and electrophysiological findings, few studies have investigated the role of the activation of specific ionotropic glutamate receptors within the PeFLH in the regulation of wake behaviors.

The first goal of this study was to elucidate the role of two ionotropic glutamate receptor subtypes within the PeFLH in the regulation of arousal-related behaviors, including locomotion, feeding and drinking, by investigating the behavioral effects of microinjections of α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) into the PeFLH in freely moving rats. The second goal of the present study was to examine the degree to which these behavioral effects are associated with, and possibly dependent on, the activation of OX neurons. This was investigated by examining c-Fos immunoreactivity (a marker of neuronal activation) in OX neurons in the PeFLH and correlating this immunoreactivity with the behavioral effects. We hypothesized that microinjections of NMDA and AMPA into the PeFLH increase arousal-related activities and that these increases are correlated with the increase in c-Fos immunoreactivity in OX neurons.

2.2. Materials and Methods

2.2.1. Animals

A total of 27 male Wistar rats (Charles River Canada, St. Constant, Quebec, Canada), weighing between 250 and 275 g at the time of surgery, were used. Of these, 13 were used for the AMPA study, and the remaining 14 for the NMDA study. Animals were housed in pairs prior to surgery, and individually after surgery, in animal care facilities with food and water available *ad libitum*. The animal rooms and behavioral testing room used in the present study were maintained on a 12:12 h light:dark cycle with lights on at 7AM and at an ambient temperature of 23°C. All handling of animals were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Dalhousie University Committee on Laboratory Animals.

2.2.2. Surgery

For implantation of guide cannulae for microinjection, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (60, 3.2, and 0.6 mg/kg, respectively, i.p.) and placed in a stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. A stainless steel guide cannula (26 gauge or 0.46 mm outer diameter; Plastics One, Roanoke, VA) was implanted unilaterally (right side) 2 mm above the PeFLH (3.1 mm posterior to bregma, 1.2 mm lateral to the midline, 6.5 mm ventral from the dura (Paxinos and Watson, 1997)). The guide cannula was fixed to the skull with an anchoring screw and dental cement. To prevent occlusion, the guide cannula was plugged with a dummy stylet of the same length.

After recovery for a minimum of 7 days after surgery, each rat was acclimatized to a behavior-testing room and an open field apparatus for 3 consecutive days during the light phase for a minimum of 2 h per day at around noon. The open field consisted of a box made of plexiglass (43.2 x 35.6 x 30.5 cm). The outer side of the plexiglass walls was covered with a black plastic sheet so that the rats were unable to see through the sides of the open field. A water bottle was placed on one wall, and several rat chow pellets were randomly placed on the floor. In the behavioral testing room, two open fields were placed adjacent to each other to test two rats concurrently. A video camera was mounted on a tripod and positioned so that a top-down view covered both open fields. During the acclimatization period, rats were subjected to frequent handling by the experimenter and mock injections consisting of the removal and replacement of the dummy stylet from the guide cannula.

2.2.3. Drugs and microinjection procedures

Two concentrations of AMPA (1 and 2 mM; Tocris, Ellisville, MO) and NMDA (1 and 10 mM; Sigma-Aldrich, Oakville, ON, Canada) in a volume of 0.1 μL were used. These doses were selected based on our pilot study as well as previous reports that these amounts of AMPA and NMDA, when injected into the basal forebrain, had behavioral but no neurotoxic effects (Page et al., 1993). All drugs were dissolved in artificial cerebrospinal fluid (ACSF), containing (in mM): NaCl (125), KCl (3), $CaCl_2$ (1.3), and $MgSO_4$ (1). All solutions were injected through a stainless steel injection cannula (33 gauge or 0.20 mm outer diameter; Plastics One) connected via polyethylene tubing (~105 cm) to a 1 μL Hamilton syringe (Hamilton, Reno, NV) driven by a syringe pump (Model

22, Harvard Apparatus, Boston, MA) at a rate of 0.1 μL/min. The tip of the injection cannula extended 2 mm beyond the end of the guide cannula.

2.2.4. Behavioral experiments

2.2.4.1. The AMPA experiment

The behavioral experiments with microinjections were carried out immediately after the 3-day acclimatization period (see above). Each rat was placed in the open field at 11 AM for additional acclimatization for about 75 min with food and water available *ad libitum*. The rat then received a microinjection of ACSF or AMPA (1 or 2 mM). For microinjection, after removal of the dummy stylet, the injection cannula was inserted into the guide cannula and 0.1 μL of the drug/vehicle was delivered at a constant rate over 1 min. The cannula was left in place for 2 min following injection to ensure diffusion of the fluid into the tissue before being removed and replaced by the dummy stylet. During this procedure, which took 4-5 min, the animal was either freely moving or gently held on a bench table near the open field. The animal was then replaced into the open field. The food pellets in the field during the acclimatization period were replaced with a fresh quantity of pre-weighed pellets. The rat's behavior in the field following the drug/vehicle injection was video-recorded for 45 minutes, and the remaining food pellets were weighed.

Each rat received a total of three injections (1 mM AMPA, 2 mM AMPA, and ACSF) with five days between injections. The order of drug treatments was randomized and no order effect was found for any behavioral measures examined ($P>0.10$). After the first and second injection sessions, animals were returned to their home cages in the animal care facility. For the last of the three sessions, the observation period after the

injection was extended to 90 min to allow for c-Fos protein synthesis, and rats were perfused immediately after this period as described below.

2.2.4.2. The NMDA experiment

The protocol was identical to that used in the AMPA experiment except that the three drug conditions entailed 1 mM NMDA, 10 mM NMDA, and ACSF.

2.2.5. Perfusion and tissue preparation

At the end of the experiments, the animals were deeply anesthetized with a ketamine/xylazine/acepromazine mixture (twice the surgical dose, i.p., see above). The descending aorta was clamped off and transcardial perfusion was conducted with 100 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.4, at room temperature), followed by 400 mL of ice-cold 4% paraformaldhehyde in 0.1 M phosphate buffer (pH 7.4). An injection cannula was inserted and secured in place during the perfusion process to facilitate identification of the injection site after tissue processing. Brains were removed and postfixed overnight at room temperature or over 2 days at 4°C. Brains were then transferred to a 30% phosphate-buffered sucrose solution for cryoprotection and stored for a minimum of 3 days at 4°C. Serial sections at 40 μm thickness were cut on a freezing microtome, and collected into 5 sets, from the genu of the corpus callosum to the nucleus of the solitary tract. One series was stained with 0.5% cresyl violet to locate injection sites and to examine possible lesions and gliosis that may have formed due to the injection procedure. Two other sets were processed for immunohistochemistry (see below).

2.2.6. Immunohistochemistry

Sections were first treated with 1% methanol and 3% hydrogen peroxide in PBS for 5 min to inactivate endogenous peroxidases, followed by three 10-min rinses in PBS. One set was processed to visualize c-Fos singly, and a second set was processed for double labeling for c-Fos and OX, as previously described (Deurveilher et al., 2006). Briefly, sections were incubated with a rabbit polyclonal anti-c-Fos antibody (1:20,000; Ab-5; catalogue No. PC38, lot No. D09803; Oncogene Research Products, Cambridge, MA) overnight at room temperature. This antibody was raised against a synthetic peptide, SGFNADYEASSSRC, which corresponds to amino acids 4-17 of human c-Fos and recognizes the ~55kDa c-Fos. Sections were then incubated for 1 h with a biotinylated donkey anti-rabbit IgG (1:1000; Jackson Laboratories-Biocan Scientific, Mississauga, ON, Canada) and reacted for 1 h in avidin-biotin-horseradish peroxidase complex (ABC, 1:500; Vectastain ABC Elite Kit; Vector Laboratories, Burlingam, CA). The c-Fos protein was visualized by reaction with 3,3'-diaminobenzidine (0.02%; DAB; Sigma-Aldrich) in the presence of nickel ammonium sulfate (0.65%; Ni) and hydrogen peroxide (0.006%) to produce a black-purple reaction product in cellular nuclei.

The second set of sections was sorted to select sections through the PeFLH and these sections were reacted for dual immunostaining for c-Fos and orexin B (OX-B). After completing the steps as above for visualizing c-Fos, the sections were incubated with a goat polyclonal anti-OX-B antibody (1:60,000; C-19; catalog No. sc-8071; lot No. E1404; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at room temperature. This antibody was raised against a 19-amino-acid peptide mapping between amino acids 60-100 of the human preproorexin precursor (Technical Services, Santa Cruz Biotechnology) and its specificity was confirmed previously (Deurveilher et al., 2006).

Sections were then incubated for 1 h in a biotinylated donkey anti-goat IgG (1:1000; Jackson Laboratories-Biocan Scientific), followed by ABC and reacted with DAB without Ni to produce a brown stain. This label was cytoplasmic and was clearly distinguishable from the black-purple nuclear staining produced by DAB-Ni for c-Fos (see Fig. 7).

Sections were then mounted, dehydrated, and coverslipped. Sections from 4-6 animals for each drug condition were processed at the same time. Sections processed without the primary antibodies were devoid of any labeling thereby confirming the specificity of the secondary antibodies used.

2.2.7. Behavioral analyses

Three exploratory (Locomotion, Rearing, Head Movement) and three non-exploratory (Grooming, Feeding, and Drinking) behaviors as well as Stillness were quantified from the video footage. The behavioral quantification was conducted by an examiner who was unaware of the treatment conditions. The examiner observed the first 10-sec bin of each 20-sec epoch. The behavior that lasted 7-10 sec during each such 10-sec bin was scored for that epoch (e.g., time spent in locomotion = 10 sec); if two behaviors lasted 4-6 sec each in a single 10-sec bin, both behaviors were scored for that epoch (e.g., time spent in locomotion = 5 sec and time spent drinking = 5 sec).

2.2.7.1. Exploratory behaviors

Exploratory behaviors included: *Locomotion* (walking, running, or turning around), *Rearing* (propped up on its hind limbs with the forepaws in contact with the sides of the open field, water bottle, or nothing at all), *and Head Movement* (stationary with the head occasionally moving with or without shifting of the body). In addition to

quantifying the time spent in exploratory behaviors, the *Distance Traveled* was measured using Ethovision software (ver. 3.0, Noldus, VA).

2.2.7.2. Non-Exploratory behaviors

Three non-exploratory behaviors were scored, including: *Grooming* (washing the face with forepaw, scratching the back of head with hindlimbs, or licking any part of the body), *Feeding* (eating food pellets), and *Drinking* (sipping from the water bottle). In addition to the amount of time spent eating, the amount of *Food Consumed* (in grams) was estimated by measuring the weight of the food pellets placed in the open field before and after the observation period. Finally, *Stillness* was scored when rats were grossly motionless (due to limitations in camera placement and resolution, it was not always possible to distinguish whether a motionless rat was in a quiet wake or asleep).

2.2.8. Histological Analyses

All histological analyses were done by an examiner who was unaware of the drug treatment conditions of the brain sections.

2.2.8.1. c-Fos immunoreactivity at the injection site

The area surrounding the injection site in which c-Fos-immunoreactive (+) neurons were concentrated was outlined in each animal after the final injection in order to delineate the area that was affected by the diffusion of ACSF, AMPA, or NMDA from the injection site. A digital image using an Axiovert 200M microscope (Carl Zeiss Canada, Toronto, ON, Canada) with a 5X objective lens was captured from a section that represented the maximum extent of c-Fos immunoreactivity surrounding the injection site for each rat. This image was then opened with Adobe Photoshop and the boundaries of c-Fos-dense area surrounding the injection site were drawn using ArtPad II (WACOM).

These boundaries were delineated by observing where the number of c-Fos+ cells tapered off from an area where they are densely packed (presence of >5 cells within a 50 μm radius to each other) to an area where the number of c-Fos+ cells is sparse (presence of only 1-3 cells within a 50 μm radius to each other) or absent.

2.2.8.2. Orexin- and c-Fos-immunoreactive neurons in the PeFLH

Neurons that were immunoreactive for both c-Fos and OX (c-Fos+/OX+), and those that were immunoreactive only for OX (c-Fos-/OX+) were counted in the PeFLH both ipsilateral and contralateral to the injection site. Four sections that spanned the rostrocaudal extent of the OX cell field (starting at 2.8 mm posterior to bregma and at 200-300 μm intervals posteriorly) were selected for cell counts. Cell counts were obtained using an Olympus microscope (BX50) coupled with a computer-based image analysis system (Neurolucida, Microbrightfield Inc., Williston, VT). In each section, the contours and anatomical landmarks of the hypothalamus at the level of the PeFLH were drawn, and both c-Fos+/OX+ and c-Fos-/OX+ neurons were plotted within the orexin cell field defined by the presence of OX+ cell bodies. Neurons were plotted with a 10X objective lens, but a 40X objective lens was used to confirm double labeling. Neurons in each section were counted bilaterally from the plots using the NeuroExplorer software program (Microbrightfield Inc.). The total cell counts from 4 sections were averaged between the two sides in each brain.

The intensity of c-Fos and OX immunostaining varied among individual neurons, and the following criteria were used for a positive label. An OX+ neuron was counted if a neuron displayed the nucleus, and a brown cellular stain was evident in the cytoplasm. OX staining was usually diffuse in appearance within the cell, but sometimes had a

granular or punctate appearance instead; both were considered positive. Positive identification of a c-Fos+ neuron required the presence of a homogeneous or punctate purple-black reaction product that was visible within the entire profile of the nucleus. Partially labeled neurons and those with light staining intensity that was judged to be equal to background levels were not plotted and excluded from the analysis.

2.2.9. Statistical Analyses

For all behavioral data, a one-way repeated measures analysis of variance (ANOVA), followed by a *post hoc* analysis (Bonferroni multiple comparisons test) if applicable, was used to determine significant differences across drug treatments. The time course of locomotor (duration and distance traveled), feeding (duration), and drinking (duration) behaviors were analyzed using a two-way repeated measures ANOVA with treatment and time as the two main factors, followed by a *post hoc* analysis (Bonferroni multiple comparisons test).

Cell count comparisons were analyzed using a non-parametric Friedman's test with treatment and side (ipsilateral versus contralateral) as the two main factors. Significant effects were further analyzed using a one-tailed Wilcoxon paired sample test.

To investigate for relationships between the percentage of c-Fos+/OX+ cells and any of the scored behaviors, a Spearman's ranked correlation analysis was used.

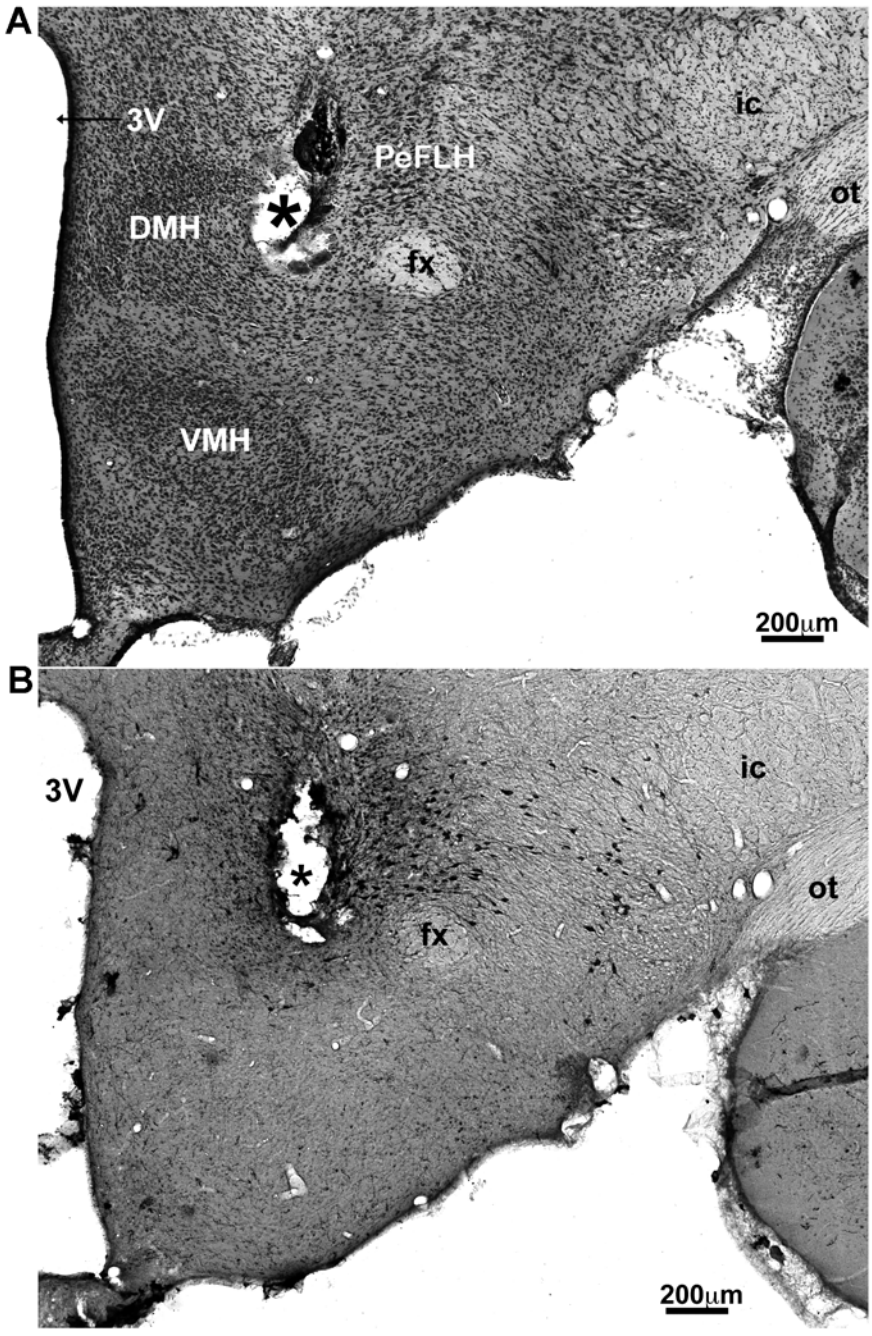
2.3. Results

2.3.1. Injection Sites

The injection sites were examined after the final (third) injection in each animal using Nissl staining in comparison with the OX staining in adjacent sections (Fig. 1). In all the rats, minimal amounts of gliosis were present in areas immediately surrounding

Figure 1

Photomicrographs of an injection site within the PeFLH. An injection site (asterisk) in the medial PeFLH shown in two adjacent sections, one Nissl stained (A) and the other immunostained for OX (B). Minimal amounts of gliosis are present in the immediate vicinity of the injection site (A), which is located within the field of OX neurons (B). The two sections were taken from the brain of the same rat that received 1 mM NMDA as the last injection. Abbreviations: 3V, third ventricle; DMH, dorsomedial hypothalamic nucleus; fx, fornix; ic, internal capsule; ot, optic tract; VMH, ventromedial hypothalamic nucleus.


A
3V
PeFLH
ic
ot
DMH
fx
VMH
200μm
B
3V
ic
ot
fx
200μm

the injection site (Fig. 1A). In both the AMPA and NMDA experiments (n=13 and 14, respectively), all the injection sites were situated around the fornix, mainly dorsally and/or medially to it, in areas that correspond to the PeFLH and contain a high concentration of OX+ neurons (Fig. 2). The injection sites for AMPA and NMDA overlapped fairly well, which justified direct comparisons of behavioral effects.

2.3.2. Behavioral Responses

2.3.2.1. AMPA Injections

2.3.2.1.1. Locomotor activity

Locomotor activity (LMA) was quantified as the total Distance Traveled and as the time spent in Locomotion; both measures provided similar results. The animals injected with ACSF in the PeFLH showed some LMA during the first 10 min, in terms of both the Distance Traveled and the time spent in Locomotion, but thereafter only showed minimum levels of LMA (Fig. 3A,B).

Analyses for the entire 45 min recording period indicated that the total Distance Traveled increased in a concentration-dependent manner ($F_{2,24}$=9.47, P<0.001; Fig. 3A1), with a significant difference between the 2 mM AMPA and ACSF conditions (+507%, P<0.001 vs ACSF). Similarly, the time spent in Locomotion over 45 min showed a concentration-dependent increase ($F_{2,24}$=9.14, P<0.001; Fig. 3B1), with a significant increase after 2 mM AMPA (+463%, P<0.001 vs. ACSF).

The time course analyses of the two LMA measures indicated that the increase in LMA following injection of either 1 or 2 mM AMPA compared to ACSF occurred within 5 min and persisted throughout the 45 min observation period, with a small decline across time (Fig. 3A2 and 3B2). The extended analyses over a 90 min period after the third injection

Figure 2

Schematic drawing showing the locations of injection sites relative to the distribution of OX neurons. Schematic drawing showing the locations of injection sites relative to the distribution of OX neurons at different rostrocaudal levels of the posterior hypothalamus (the numbers on the left indicate the distance posteriorly (P) from bregma in mm). The left column shows plots of OX neurons from a representative rat. The large solid circles and squares indicate the estimated locations of injection sites for AMPA (middle column, n=13) and NMDA (right column, n=14), respectively, based on the last injection in each animal.

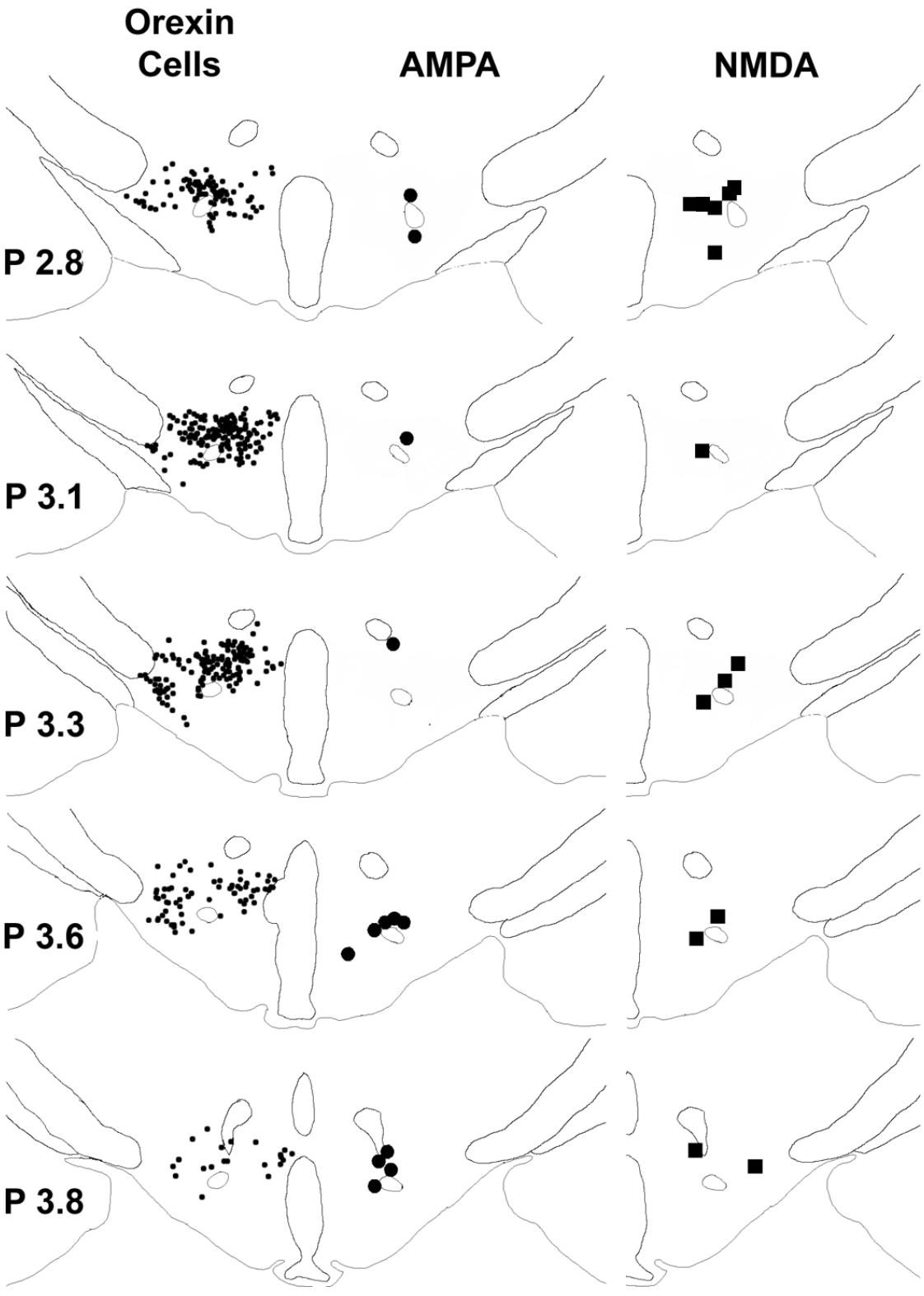
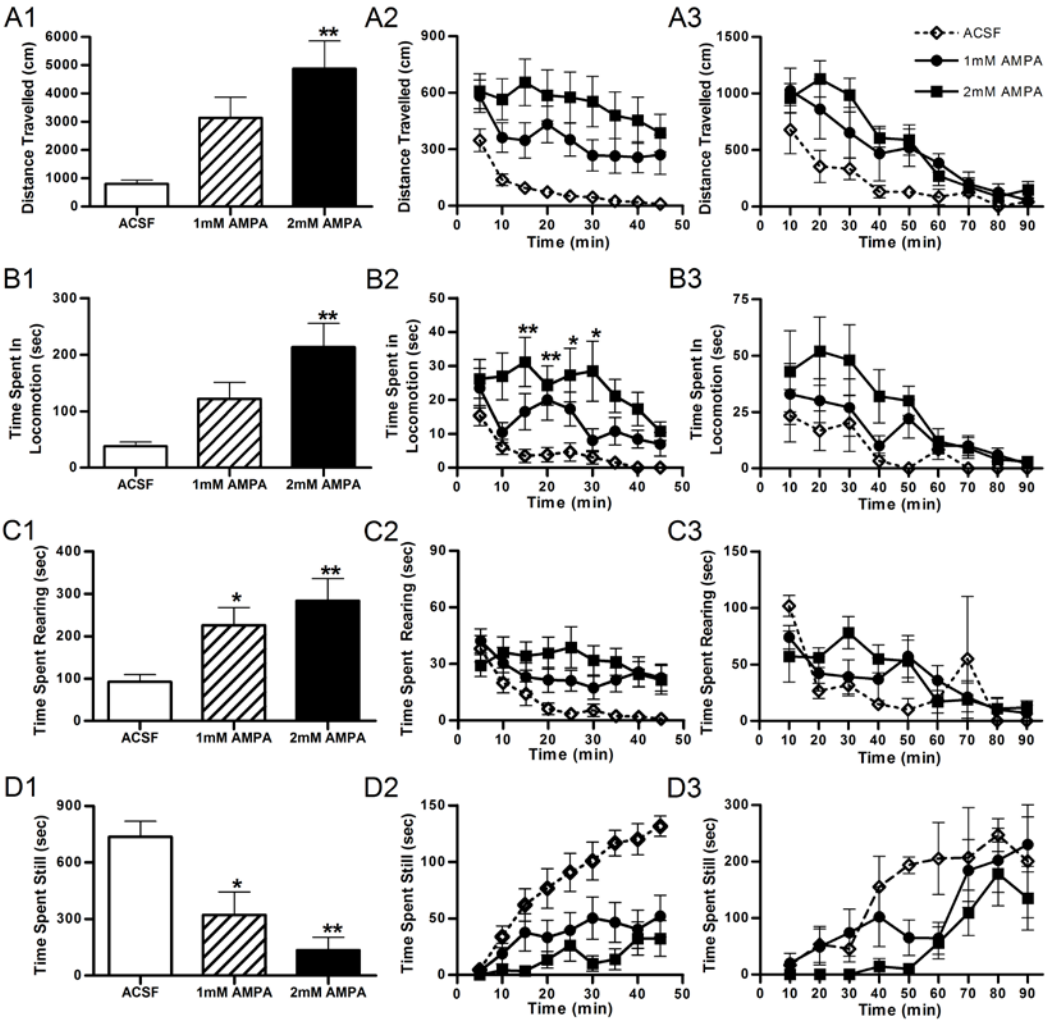

Orexin Cells
AMPA
NMDA
P 2.8
P 3.1
P 3.3
P 3.6
P 3.8

Figure 3

Distance Traveled, and times spent in Locomotion, Rearing, and Stillness following administration of ACSF, 1 mM AMPA, or 2 mM AMPA. Values (mean ± S.E.M., cm or sec) for Distance Traveled (A), and times spent in Locomotion (B), Rearing (C), and Stillness (D) following administration of ACSF, 1 mM AMPA, or 2 mM AMPA. Values are presented as a total over the 45 min period (left, A-D1; n=13), a time course across the 45 min period (middle, A-D2; n=13), and a time course across the 90 min period after injection (right, A-D3; n=3 for ACSF, and n=5 for each concentration of AMPA). Each rat received a total of three injections with ACSF and low and high concentrations of AMPA in a random order. * $p<0.05$ versus ACSF; ** $p<0.01$ versus ACSF.


A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
Distance Travelled (cm)
Time Spent In Locomotion (sec)
Time Spent Rearing (sec)
Time Spent Still (sec)
ACSF
1mM AMPA
2mM AMPA
Time (min)

(to allow for full c-Fos expression) showed that the AMPA effects on LMA disappeared by 60-70 min after injection (Fig. 3A3 and 3B3).

2.3.2.1.2. Rearing

A concentration-dependent increase in Rearing was seen over the 45 min observation period ($F_{2,24}$=6.77, P<0.005; Fig. 3C1) following injections of 1 and 2 mM AMPA (+146% and +209%, respectively; P<0.05 for 1 mM AMPA vs. ACSF, P<0.01 for 2 mM AMPA vs. ACSF). This increase was maintained through the first 45 min (Fig. 3C2) and disappeared by 60-70 min post-injection (Fig. 3C3).

2.3.2.1.3. Stillness

In contrast to the increases in LMA and Rearing after AMPA injections, there was a concentration-dependent decrease in the time spent in Stillness over the 45 min observation period ($F_{2,24}$=11.21, P < 0.0005; Fig. 3D1) following injections of 1 and 2 mM AMPA (-56% and -82%, respectively; P<0.05 for 1 mM AMPA vs. ACSF, P<0.001 for 2 mM AMPA vs. ACSF). After AMPA injections, the time spent in Stillness increased more slowly and remained at lower levels when compared to ACSF injections over 45 min (Fig. 3D2), but reached similar levels to those under ACSF condition by 70-80 min (Fig. 3D3).

2.3.2.1.4. Head Movement and Grooming

There were no significant changes in the time spent in Head Movement ($F_{2,24}$=2.02; NS) or Grooming ($F_{2,24}$=1.71; NS) following either concentration of AMPA compared to ACSF (Table 1).

Table 1

Total durations of time spent in Grooming or in Head Movement during the 45-min observation period after administration of ACSF, AMPA or NMDA. Total durations of time (mean ± S.E.M. sec) spent in Grooming or in Head Movement during the 45-min observation period after administration of ACSF, AMPA or NMDA. Each animal received three microinjections with ACSF plus low and high concentrations of either drug, in a random order. AMPA, n=13; NMDA, n=14. There were no significant differences between treatment conditions in either the AMPA or the NMDA experiment.

Behavior	AMPA			NMDA		
	ACSF	1 mM	2 mM	ACSF	1 mM	10 mM
Grooming (sec)	173 ± 32	136 ± 30	100 ± 26	194 ± 21	217 ± 21	167 ± 32
Head Movement (sec)	217 ± 34	319 ± 52	323 ± 46	359 ± 35	396 ± 34	360 ± 27

2.3.2.1.5. Feeding and Drinking

A trend of an increase in the amount of food consumed was seen over 45 min following 2 mM AMPA injections (+180% vs. ACSF; $F_{2,24}$=2.99; P=0.069; Fig. 4A).

The amount of time spent in Drinking increased following injection with 1 and 2 mM AMPA compared to ACSF ($F_{2,24}$=4.23, P<0.05; Fig. 4C) and this increase was significant following 2 mM AMPA (+745%; P<0.05 vs. ACSF). The time course analysis showed that the increase in drinking behavior lasted for 50-60 min post-injection (data not shown).

2.3.2.2. NMDA Injections

In general, the behavioral effects of 1 or 10 mM NMDA injections were smaller in magnitude and lasted for shorter periods of time compared to 1 or 2 mM AMPA injections, and any changes returned to control (ACSF) levels within the 45 min of observation period. In addition, for most behavioral measures, the NMDA effects were clearly seen, and in some cases significant, only at the higher concentration (10 mM).

2.3.2.2.1. Locomotor activity

As observed in the AMPA experiment, the two measures of LMA, i.e., time spent in Locomotion and Distance Traveled, provided similar results. The time spent in locomotion during the 45 min observation period significantly increased ($F_{2,26}$=4.48, P<0.05; Fig. 5B1) following 10 mM NMDA in comparison to ACSF (+217%, P<0.05 vs ACSF). Likewise, the Distance Traveled over 45 min was greater ($F_{2,26}$=3.88, P<0.05; Fig. 5A1), albeit non-significantly, following 10 mM NMDA (+184%) than ACSF.

The time course analysis of LMA during the 45 min period indicated that the increase in both Distance Traveled and time spent in Locomotion following 10 mM

Figure 4

Amounts of food consumed and time spent Drinking following microinjections of AMPA and NMDA. Amounts of food consumed following microinjections of AMPA (A) and NMDA (B), and times spent Drinking following microinjections of AMPA (C) and NMDA (D), all in a 45-minute period. Each rat received three injections with ACSF and low and high concentrations of NMDA or AMPA in a random order. AMPA, n = 13; NMDA, n = 14. *p<0.05 versus ACSF; **p<0.001 versus ACSF.

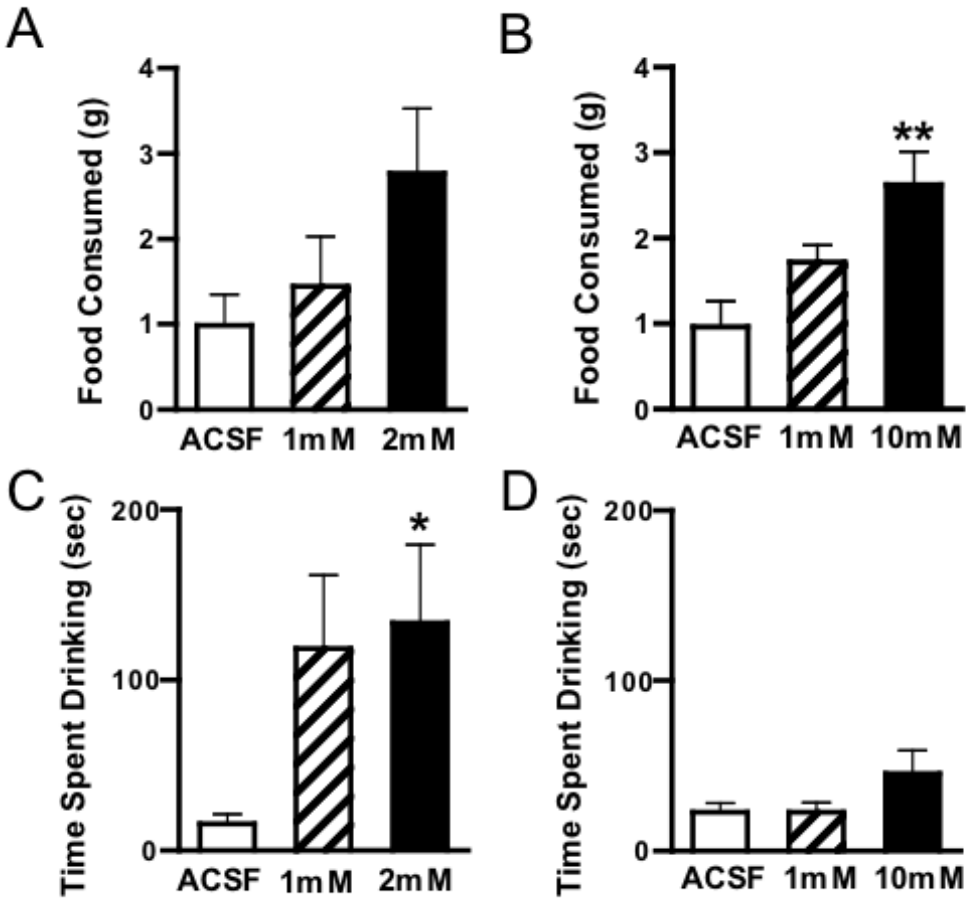
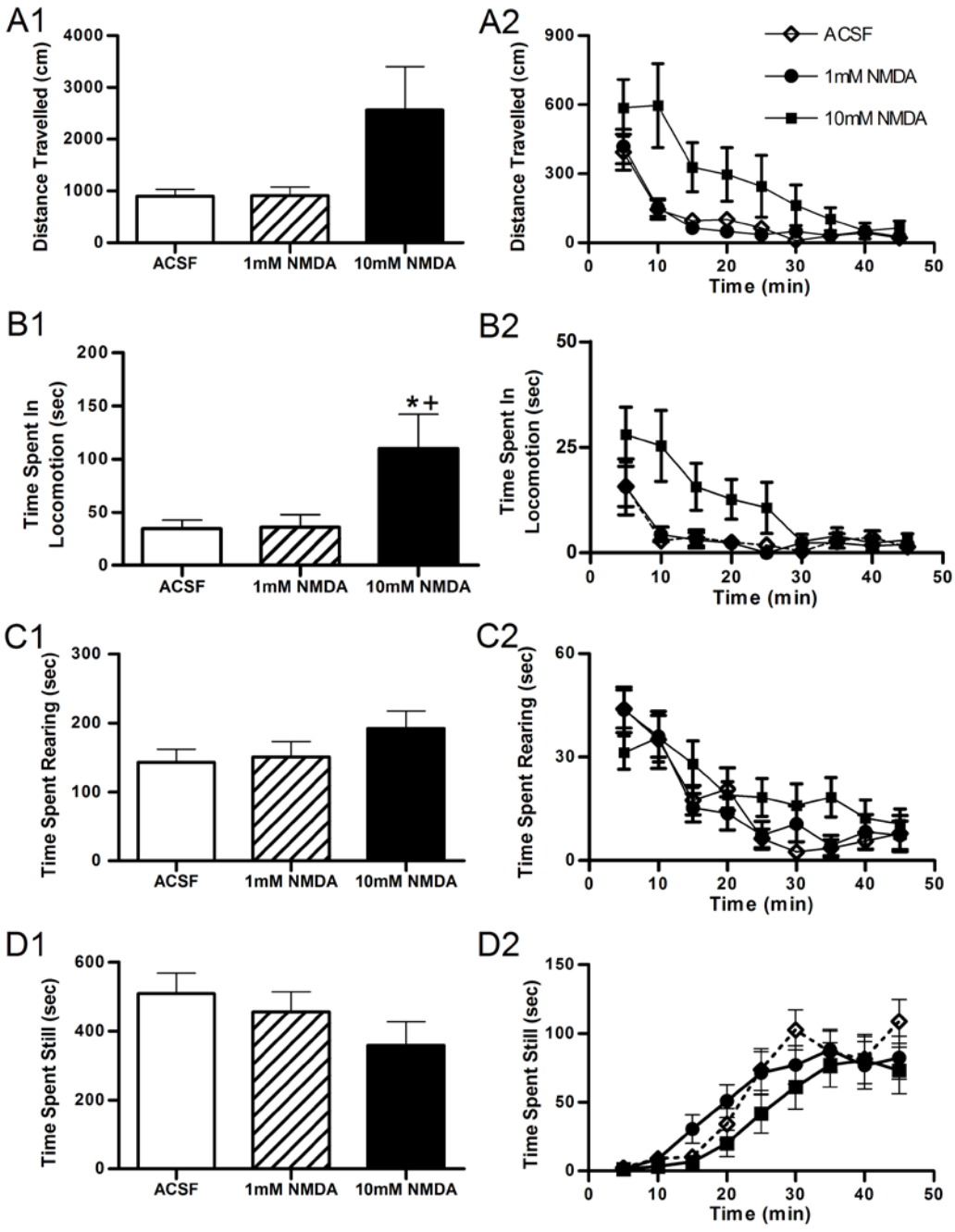

A
Food Consumed (g)
4
3
2
1
0
ACSF
1mM
2mM
B
Food Consumed (g)
**
ACSF
1mM
10mM
C
Time Spent Drinking (sec)
200
100
0
*
ACSF
1mM
2mM
D
Time Spent Drinking (sec)
200
100
0
ACSF
1mM
10mM

Figure 5

Distance Traveled, and times spent in Locomotion, Rearing, and Stillness following administration of ACSF, 1 mM NMDA, or 10 mM NMDA. Values (mean ± S.E.M., cm or sec) for Distance Traveled (A), and times spent in Locomotion (B), Rearing (C), and Stillness (D) following administration of ACSF, 1 mM NMDA, or 10 mM NMDA. Values are presented as a total (left, A-D1) and as a time course (right, A-D2). For each condition, n=14. Each rat received three injections with ACSF and low and high concentrations of NMDA in a random order. *$p<0.05$ versus ACSF; +$p<0.05$ versus 1mM NMDA.


A1
Distance Travelled (cm)
4000
3000
2000
1000
0
ACSF
1mM NMDA
10mM NMDA
A2
Distance Travelled (cm)
900
600
300
0
ACSF
1mM NMDA
10mM NMDA
0
10
20
30
40
50
Time (min)
B1
Time Spent In Locomotion (sec)
200
150
100
50
0
*+
ACSF
1mM NMDA
10mM NMDA
B2
Time Spent In Locomotion (sec)
50
25
0
0
10
20
30
40
50
Time (min)
C1
Time Spent Rearing (sec)
300
200
100
0
ACSF
1mM NMDA
10mM NMDA
C2
Time Spent Rearing (sec)
60
30
0
0
10
20
30
40
50
Time (min)
D1
Time Spent Still (sec)
600
400
200
0
ACSF
1mM NMDA
10mM NMDA
D2
Time Spent Still (sec)
150
100
50
0
0
10
20
30
40
50
Time (min)

NMDA occurred mostly during the first 10-15 min after injection (Fig. 5A2 and 5B2, respectively) and this increase lasted no longer than 30 min.

2.3.2.2.2. Rearing and Stillness

The injection of NMDA at 10 mM resulted in a trend for an increase in the time spent in Rearing ($F_{2,26}$=2.89, P=0.07; Fig. 5C1 and 2) and a trend for a decrease in the time spent in Stillness ($F_{2,26}$=3.06, P=0.06; Fig. 5D1 and 2).

2.3.2.2.3. Head Movement and Grooming

As observed in the AMPA experiment, there were no significant changes in the time spent in Head Movement ($F_{2,26}$=0.59, NS) or Grooming ($F_{2,26}$=1.14, NS) following either 1 or 10 mM NMDA (Table 1).

2.3.2.2.4. Feeding and Drinking

Food consumption over the 45 min observation period significantly increased ($F_{2,26}$=9.02, P<0.001; Fig. 4B) following treatment with 10 mM NMDA compared to ACSF (+160%, P<0.001 vs. ACSF). Although most Feeding occurred during the first 15-30 min post-injection, some rats were observed to feed closer to the end of the 45 min period (data not shown). The increase in Feeding after 10 mM NMDA was accompanied by a non-significant increase in Drinking ($F_{2,26}$=1.93, NS; Fig. 4D).

2.3.3. c-Fos Immunoreactivity

2.3.3.1. Areas Surrounding the Injection Site

As an estimate for the area that was most likely directly affected by the drug injections, we examined c-Fos immunoreactivity around the injection site in the PeFLH. Neurons that were c-Fos+ were visible and densely packed in areas immediately surrounding the injection site; the labeling was progressively sparser and lighter further

away from the injection site. It was thus possible to delineate this dense area of c-Fos immunoreactivity by eye. The boundaries of dense c-Fos immunoreactivity were traced and overlaid for comparison among the treatment groups (Fig. 6). The area containing dense c-Fos immunoreactivity after ACSF injections was approximately 750 μm wide mediolaterally (Fig. 6 top). After AMPA injections, the mediolateral extent of dense c-Fos immunoreactivity was approximately 1500 μm, regardless of the concentration used (1 or 2mM; Fig. 6 middle left and bottom left). The mediolateral extent of c-Fos immunoreactivity after injections of either concentration of NMDA (1 or 10mM) was about 1100 μm (Fig. 6 middle right and bottom right), which was intermediate between ACSF and AMPA injections.

2.3.3.2. c-Fos+/OX+ Neurons in the PeFLH

c-Fos+/OX+ cells were intermingled with both OX+ neurons without c-Fos immunoreactivity and Fos+ neurons without OX+ immunoreactivity in the PeFLH (Fig. 7), with a higher concentration of c-Fos+/OX+ and Fos+ neurons near the injection site. The total numbers of OX+ neurons, regardless of c-Fos immunoreactivity, did not differ between ACSF and drug conditions in either the AMPA ($F_{2,11}$=1.08; NS) or the NMDA experiment ($F_{2,13}$=0.11, NS; Table 2), suggesting that similar populations of OX+ neurons were examined across all the conditions. The number of c-Fos+/OX+ neurons was also analyzed as a percentage of the total number of OX+ cells (Table 2), and the analyses of this measure revealed several significant differences between treatment groups as described below.

Figure 6

Estimated extents of areas that were affected by ACSF, AMPA, and NMDA injections, as revealed by c-Fos immunoreactivity. Estimated extents of areas that were affected by ACSF, AMPA, and NMDA injections, as revealed by c-Fos immunoreactivity. Tracings from the section representing the widest spread of Fos+ neurons from each rat were superimposed using the ventral tip of the injection cannula tract as the reference point. Solid lines outline the location of injection cannula tract. Dotted lines outline the border of areas containing dense Fos+ neurons (see the Methods for details).

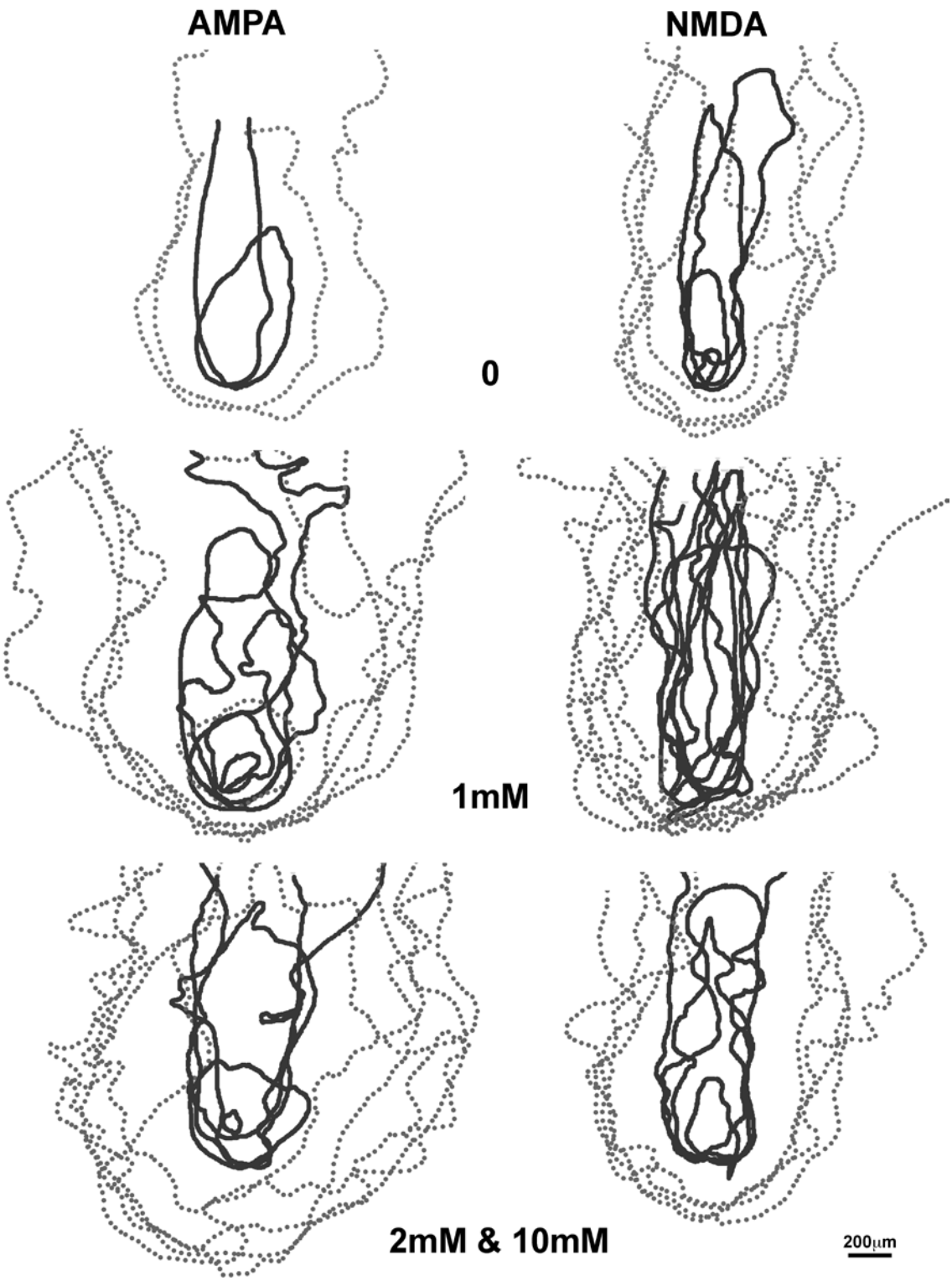

AMPA
NMDA
0
1mM
2mM & 10mM
200μm

Figure 7

Micrograph of a section double immunostained for c-Fos and OX. Micrograph of a section double immunostained for c-Fos (black, nuclear) and OX (grey, cytoplasmic) in a rat injected with 1 mM AMPA 90 min before perfusion. Black arrowheads indicate neurons that are Fos+/OX-; white arrows indicate neurons that are Fos-/OX+; black arrows indicate neurons that are Fos+/OX+.

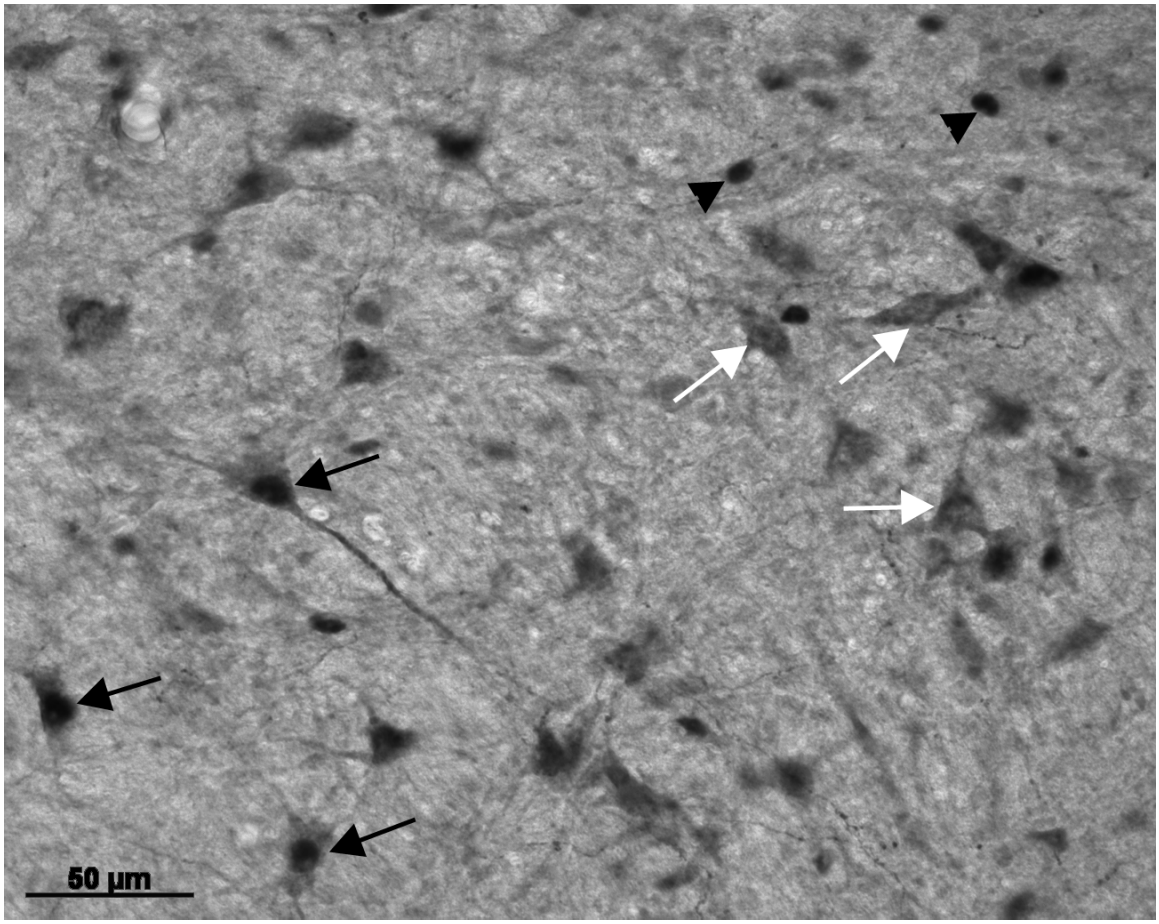

50 µm

Table 2

Percentage of OX+/Fos+ cells and the total number of OX+ cells in the PeFLH on the side ipsilateral or contralateral to the last microinjection in each rat. Percentage (mean ± S.E.M.) of OX+/Fos+ cells and the total number of OX+ cells on the side ipsilateral or contralateral to the last microinjection in each rat. Four sections were used for each treatment condition and the number of animals examined in each condition is indicated in parentheses. *$p<0.05$, ipsilateral versus contralateral side.

		AMPA			NMDA		
		ACSF (3)	1 mM (4)	2 mM (4)	ACSF (4)	1 mM (6)	10 mM (4)
OX+/Fos+ (%)	Ipsilateral	37.6 ± 13.0	55.4 ± 7.9 *	56.3 ± 12.7*	16.3 ± 7.5	15.9 ± 3.4	14.6 ± 2.6
	Contralateral	29.7 ± 8.9	33.4 ± 9.0	25.1 ± 10.0	12.8 ± 5.2	9.1 ± 2.4	4.2 ± 3.4
Total	Ipsilateral	350.7 ± 11.0	316.8 ± 48.3	267.8 ± 64.2	416.8 ± 23.1	414.3 ± 38.0	389.5 ± 71.3
	Contralateral	366.3 ± 62.5	296.0 ± 28.7	323.5 ± 37.8	441.3 ± 36.5	408.0 ± 61.3	430.5 ±102.1

2.3.3.2.1. AMPA Injections

As shown in Table 2, injections of 1 and 2 mM AMPA induced a significant increase in the percentage of c-Fos+/OX+ cells on the ipsilateral side compared to the contralateral side (Treatment x Side: $\chi_r^2{}_{6,4}$=10.81; P<0.05; ipsi > contra, P<0.05 for 1 mM & 2 mM AMPA). On the ipsilateral side, the percentage of c-Fos+/OX+ cells tended to be increased following injections of 1 mM (+47%) and 2 mM (+50%) compared to ACSF. On the contralateral side, the percentages of c-Fos+/OX+ neurons after 1 and 2 mM AMPA were similar to the percentage of double labeled neurons on the ipsilateral/contralateral side following ACSF injection.

2.3.3.2.2. NMDA Injections

In contrast to AMPA, injections of 1 and 10 mM NMDA did not significantly affect the percentage of c-Fos+/OX+ labeling compared to the ACSF condition in either the ipsilateral or contralateral side (($\chi_r^2{}_{6,4}$=7.17, NS; Table 2). It is of note that the cell counts after ACSF injection for the AMPA injected group is higher than the NMDA injected group. We believe this discrepancy to be the result of a variability in the immunostaining technique. AMPA and NMDA experiments were completed at different time points and the OX-B antibody used were from two different lots. Despite this, there is a consistency in the OX counts between the treatment conditions within each experiment group, making it still possible to compare the percentage of c-Fos+/OX+ labeling, between treatment conditions and on the ipsi- and contralateral sides, in the AMPA and NMDA experiments separately.

2.3.4. Correlations Between c-Fos+/OX+ Labeling and Behavioral Measures

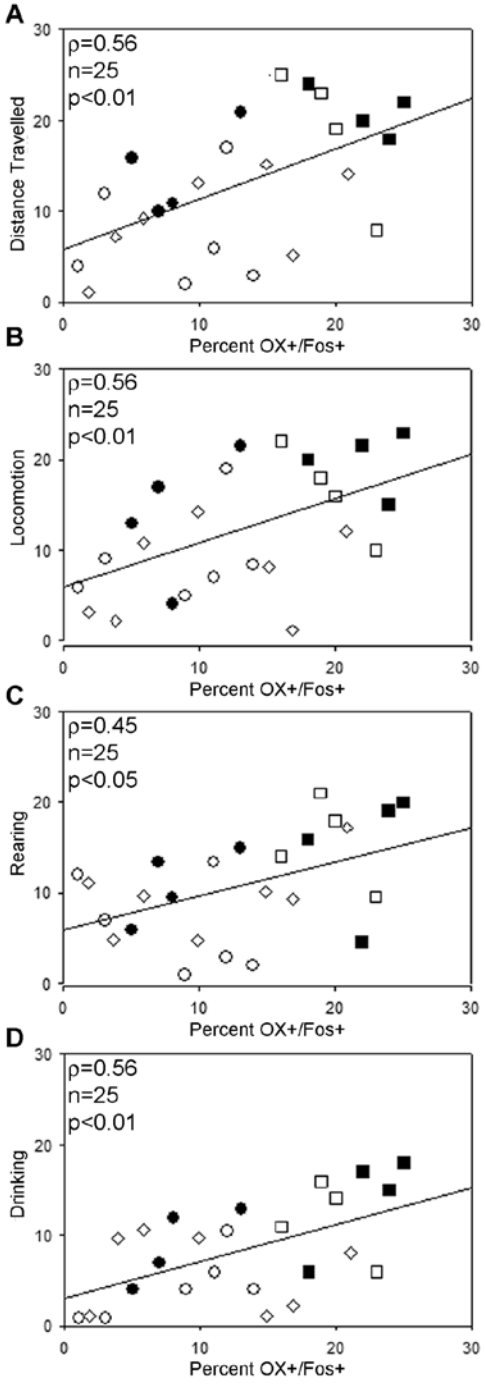
To determine whether the c-Fos immunoreactivity of OX neurons had any role in the behavioral effects of AMPA/NMDA injections, we combined the data from the AMPA and NMDA experiments, and examined the correlation between the percentage of double labeled OX neurons and the measures of behavioral activation that showed a significant increase following NMDA or AMPA treatments, including: Distance Traveled, times spent in LMA, Rearing, Drinking and Feeding. A moderate but significant correlation was seen between the percentage of c-Fos+/OX+ cells vs. Distance Traveled (ρ=0.56; P<0.01; n=25; Fig. 8A), or time spent in Locomotion (ρ =0.53; P<0.01; n=25; Fig. 8B), Rearing (ρ =0.45; P<0.05; n=25; Fig. 8C), and Drinking (ρ =0.56; P<0.01; n=25; Fig. 8D). There was no significant correlation between the percentage of c-Fos+/OX+ cells and Feeding (ρ =0.147; P=0.47). Figure 7 also shows that, overall, AMPA produced a higher level of behavioral activity along with more activation of OX neurons compared to NMDA.

2.4. Discussion

The present study showed that AMPA and NMDA, when injected into the PeFLH, elicited an increase in arousal-related behaviors including locomotion, rearing, feeding, and drinking. Overall, AMPA had larger and longer-lasting effects than NMDA on most behaviors, while NMDA yielded more consistent effects, particularly on feeding. The amounts of these behaviors except feeding were positively correlated with the activity levels of OX neurons as assessed by c-Fos immunoreactivity.

Figure 8

Correlations between OX+/Fos+ cell counts and time spent in Locomotion, Distance Traveled, time spent Rearing, and time spent in Drinking after microinjections of ACSF, AMPA, and NMDA. Relationships between OX+/Fos+ cell counts and time spent in Locomotion (A), Distance Traveled (B), time spent Rearing (C), and time spent in Drinking (D) during 45 min period after microinjections of ACSF (diamond), 1 mM AMPA (white circle), 2 mM AMPA (black circle), 1 mM NMDA (white square), and 10 mM NMDA (black square). Correlation coefficients were obtained using a Fisher's r to Z test. For each behavior, n = 25. Moderate but significant correlations are seen between the OX+/Fos+ cell counts and all 4 behaviors.


A
ρ=0.56
n=25
p<0.01
Distance Travelled
Percent OX+/Fos+
B
ρ=0.56
n=25
p<0.01
Locomotion
Percent OX+/Fos+
C
ρ=0.45
n=25
p<0.05
Rearing
Percent OX+/Fos+
D
ρ=0.56
n=25
p<0.01
Drinking
Percent OX+/Fos+

2.4.1. Technical Considerations

In the present study, each rat received 3 injections at 5-day intervals and in a randomized order, including a low and a high dose of either AMPA (1 or 2 mM) or NMDA (1 or 10 mM), and the vehicle. Although this type of experimental design is commonly used to reduce between-subject variability for comparing behavioral responses under multiple conditions, repeated injections into the same brain region could affect the viability of neurons around the injection site and, consequently, behavioral responses to subsequent injections. This is, however, unlikely to be an issue in the present study because there was only minor gliosis around the injection site and, furthermore, there were no order effects on behavioral responses under any of the drug conditions.

Another methodological issue regards estimation of food consumption. We used regular rat chow and estimated food consumption by comparing the weight of pellets at the time of injection and after the observation period. Rats, however, often dropped crumbles of pellets on the floor while feeding, and we did not correct for spillage. However, the amount of spillage was probably negligible because the food consumption in the present study is, in fact, comparable to that reported in studies that used food preparations (e.g., milk-mash), which provide more accurate measures of food consumption, to study the effects of injections of OX or glutamate and its agonists into the PeFLH in food-satiated rats (Stanley and Thomas, 1993; Doane et al., 2007).

Finally, the hunger level at the time of drug/vehicle injection was not directly controlled in individual rats in this study. Nonetheless, with food and water available *ad lib*, all rats would be expected to be food-satiated to a level that is consistent with the

time of day of testing (around the middle of the light phase). This condition could set a certain threshold for an overt feeding response to occur, whereas a ceiling effect would be possible only as a result of physiological constraints of the gastrointestinal system because food and water were available freely during testing.

2.4.2. Behavioral Responses to AMPA and NMDA Microinjections

In the present study, injections of AMPA and NMDA into the PeFLH elicited dose-dependent increases in specific, natural behaviors, including LMA, rearing, feeding and drinking. AMPA was generally more effective than NMDA, at least at the amounts used.

LMA was significantly increased upon the administration of the higher concentration of either AMPA (2 mM) or NMDA (10 mM), while rearing was increased only after injection of the higher concentration (2 mM) of AMPA. These results are consistent with previous reports that electrical stimulation of the PeFLH (Parker and Sinnamon, 1983; Sinnamon and Stopford, 1987; Lammers et al., 1988) or, administration of glutamate receptor agonists or GABA receptor antagonists into the PeFLH produced a broad range of LMA, from increased general locomotion to flight-like locomotor responses (Shekhar et al., 1987; Marciello and Sinnamon, 1990).

Feeding was increased to a similar extent after injections of 2 mM AMPA and 10 mM NMDA into the PeFLH, but this increase was significant only for NMDA, likely because of the larger variability in the AMPA condition. These results are consistent with previous studies reporting that NMDA was generally more effective than AMPA in eliciting feeding when injected into the PeFLH (Stanley et al., 1993a; Stanley et al., 1993b; Duva et al., 2002). In addition, these studies reported that stronger and more

consistent responses were present following injections to the lateral field of the PeFLH. In the present study, both AMPA and NMDA were injected into the medial field of the PeFLH, and it is possible that the observed increase in feeding activity was due to diffusion of the drugs into the lateral PeFLH.

Drinking behavior was increased following microinjections of 1 and 2 mM AMPA and, to a much lesser extent, 10 mM NMDA, into the medial PeFLH. Consistent with our results, electrolytic and excitotoxic lesions of this area produced adipsia (Huang and Mogenson, 1974; Clark et al., 1991), whereas electrical stimulation of the PeFLH immediately lateral to the DMH or VMH resulted in dipsogenesis (Greer, 1955; Mogenson et al., 1971). In addition, a previous study has shown that administration of glutamate or glutamate receptor agonists into the lateral PeFLH did not initiate water consumption (Stanley et al., 1993a). Collectively, these results suggest a dipsogenic role for glutamatergic afferents to the medial PeFLH. Alternatively, increased drinking may be secondary to increased feeding following AMPA and NMDA injections.

PeFLH receives inputs from many regions of the brain, including those neurons containing glutamate (see Introduction). Within the hypothalamus, two likely sources of glutamate are local glutamate interneurons within the PeFLH (Li et al., 2002), which have been shown to serve as an intermediary for communication from an OX neuron to another OX neuron, and putative glutamatergic neurons in the DMH (Chou et al., 2003), a nucleus implicated in the regulation of circadian rhythms and wake related behaviors such as feeding and locomotor activity. Extrahypothalamic sources of glutamate afferents to the PeFLH include the ascending projections from the lateral parabrachial nucleus, involved in visceral and other sensory processing (Niu et al., 2010), and reticular

neurons in the brainstem (Kaneko et al., 1989; Jones, 1995), which are involved in wakefulness and related behaviors. The PeFLH also receives descending glutamatergic projections from the basal forebrain (Henny and Jones, 2006) and the medial prefrontal cortex (Vertes, 2004). While some of these glutamatergic afferents have been demonstrated to project directly to OX cells (Yoshida et al., 2006; Niu et al., 2010), their connections to other cell types within this area have not been fully investigated. Studies on the role of these specific afferents in the elicitation of the various physiological behaviors will serve to further our understanding of glutamatergic regulation of arousal-related behaviors in this region.

2.4.3. Role of OX Neurons in Behavioral Responses Elicited by AMPA and NMDA

We found that OX neurons increased c-Fos levels following intra-PeFLH injections of AMPA, but not NMDA. Furthermore, the level of c-Fos expression was correlated with the amounts of exploratory and drinking behaviors when both drug treatment groups were combined. These results suggest that OX neurons could have been involved in eliciting these behavioral responses to AMPA and NMDA injections.

The positive correlations between the number of c-Fos+/OX+ cells and exploratory behaviors is consistent with previous findings that increased locomotion was accompanied by increased discharge in OX neurons (Mileykovskiy et al., 2005) and increased OX levels in the brain (Kiyashchenko et al., 2002) and cerebrospinal fluid (Wu et al., 2002). OX neurons also increased c-Fos expression during active wake, but not during quiet wake (Torterolo et al., 2003). OX neurons may increase LMA through their projections to many nuclei of the wake system, including the TMN and LC (Nambu et al., 1999). Consistent with this possibility, we observed an increase in c-Fos

immunoreactivity bilaterally in the TMN and LC after AMPA administration into the PeFLH. Further support is provided by findings that OX or OX neurons activated histaminergic neurons in the TMN (Huang et al., 2001) and noradrenergic neurons in the LC (Horvath et al., 1999), and that administration of OX directly into the LC induced hyper-locomotion and rearing activity in rats (Hagan et al., 1999).

We also found a positive correlation between the number of OX neurons expressing c-Fos and drinking behavior. This is consistent with an increased water intake after central administration of OX (Kunii et al., 1999). Anatomically, the PeFLH receives a direct input from the subfornical organ, a well-documented site for osmotic regulation (Lind et al., 1984), which is also reciprocally innervated by OX neurons (Ono et al., 2008). The present study supports the role of medially-located OX neurons in drinking.

There was no correlation between feeding and the number of c-Fos+/OX+ cells, which seems surprising in light of the role of OX in feeding behavior. For example, the number of OX immunoreactive cells in the PeFLH is known to be increased during fasting (Akiyama et al., 2004), feeding is increased after intracerebroventricular administration of OX (Sakurai et al., 1998; Edwards et al., 1999; Sweet et al., 1999), and OX-induced feeding requires activation of the NMDA receptor (Doane et al., 2007). However, as mentioned above, feeding responses to glutamate and its receptor agonists were strongest when the drugs were administered into the lateral PeFLH (Stanley et al., 1993b). Furthermore, OX neurons that expressed c-Fos after feeding following food deprivation were located primarily in this area (Harris et al., 2005). Therefore, the lack of correlation between feeding and the activation of OX neurons in the present study might be due to the medial locations of drug injections in the present study.

AMPA was more effective than NMDA when injected into the PeFLH in increasing c-Fos levels in OX neurons, as well as in eliciting behavioral responses. This is consistent with a greater depolarization and firing of OX neurons *in vitro* in response to AMPA compared to NMDA; AMPA elicited greater responses than NMDA administered at 2-3 fold higher concentrations . It is likely that NMDA, at the amounts used in the present study, was unable to maintain a sustained activity of OX neurons that would be required to elicit robust and long-lasting behavioral effects as observed after AMPA injections. In fact, the brief behavioral activation even after using our highest amount of NMDA was followed by a long (at least one hour) period of increased Stillness similar to following ACSF injection. Wake-induced c-Fos levels are known to decline within an hour of sleep (Basheer et al., 1997); therefore, it is plausible that any c-Fos immunoreactivity induced by a transient increase in LMA would have returned to baseline levels by the time of perfusion (i.e., 90 minutes after injection).

While we showed a positive correlation between OX cell activity and arousal-related behaviors, it is likely that non-OX neurons in the PeFLH were also recruited to elicit these behaviors, as evidenced by the abundance of c-Fos+/OX- neurons mixed with OX neurons following AMPA and NMDA injections. These c-Fos labeled neurons may include MCH-containing neurons (Bittencourt et al., 1992; Hahn, 2010), glutamate interneurons (Li et al., 2002), and probably other neurons. Of these, MCH-containing neurons are of particular interest because their role is similar to that of OX neurons for food consumption (Qu et al., 1996) but different for locomotion and sleep-wake regulation. MCH has been associated with inhibition of locomotor responses (Elias et al., 2008) and implicated in the regulation of rapid eye movement sleep (Verret et al., 2003).

Experiments involving selective activation of either of these two distinct populations would help determine the specific roles of these cell populations in the observed behavioral responses.

2.4.4. Conclusions

We have demonstrated that the activation of both AMPA and NMDA receptors in the medial PeFLH increased locomotor and rearing activities. In addition, drinking activity was preferentially elicited with AMPA receptor activation, while feeding activity was more effectively elicited by NMDA receptor activation. With the exception of feeding, all these behaviors were positively correlated with the percentage of activated OX cells. These results suggest that both AMPA and NMDA receptor subtypes are differentially involved in glutamatergic stimulation of specific-arousal related behaviors and support the role of glutamatergic regulation of OX cells within the PeFLH on locomotive and ingestive behaviors.

CHAPTER 3: DISCUSSION

3.1. Future Studies

The present study demonstrated a role for glutamate within the PeFLH in the regulation of LMA and ingestive behaviors, and also showed a positive correlation between the activity of OX neurons and these behaviors. The study left two points unanswered and these points can be addressed with additional experiments. The first point pertains to the need to identify the endogenous sources of glutamate to the PeFLH, or to specific neuronal populations within this region, while the second point regards the use of more selective means of activating specific neuronal populations within the PeFLH.

3.1.1. Approaches For Identifying Glutamatergic Inputs to the PeFLH

While afferents to the PeFLH (Hahn and Swanson, 2010) and, more specifically, to OX neurons have already been identified (Yoshida et al., 2006), the neurotransmitter phenotypes of only a handful of these inputs have been characterized, with even fewer of these being identified as glutamatergic (see Discussion in Chapter 2). Identifying all glutamate inputs to the PeFLH or to specific neuronal populations, such as OX or MCH neurons, will help further our understanding of how other areas of the brain use glutamate to modulate neuronal activity within the PeFLH to regulate arousal behaviors.

One method to identify glutamate afferents would be to utilize a triple label anatomical tracing study. Afferents can be first labeled with an anterograde tracer (i.e. phaseolus vulgaris-leucoagglutinin) that is injected into an area that is a likely candidate to project to the PeFLH as demonstrated in previous studies (Yoshida et al., 2006; Hahn and Swanson, 2010) and is suspected to be glutamatergic. The second label would label

axonal terminals for vesicular glutamate transporter (VGLUT) 1 or 2, glutamate neuronal markers known to be expressed in this region (Collin et al., 2003; Hisano, 2003; Rosin et al., 2003). If our suspected afferent source is glutamatergic, one would expect to see a colocalization of our tracer with VGLUT 1 or 2 within the PeFLH. The third label to be used would be specific to a particular neuronal phenotype, such as OX or MCH, which would show the neuronal population of interest to be receiving these glutamatergic afferents.

This triple labeling technique would help to reveal glutamatergic inputs from various areas of the brain to specific neuronal populations within the PeFLH, It would also show whether subpopulations of a particular neuronal group would receive larger amounts of afferent inputs preferentially from one or more distant brain regions (discussed below). Once a glutamatergic afferent has been identified, one could electrically or chemically stimulate the source of this afferent while simultaneously measuring the amount of glutamate released within the PeFLH using a glutamate biosensor. Although this technique is not capable of measuring glutamate release at a synapse, it can still provide further insight regarding how these distant brain regions might cause glutamate release in this area to regulate behavioral responses.

3.1.2. Approaches for Selective Activation of Neuronal Populations Within the PeFLH

I mentioned above that many c-Fos+/OX- cells were also present around the injection site. This suggests the possibility that the behaviors elicited by AMPA or NMDA injections were the result of stimulation of not only OX but also non-OX neurons. Some of these non-OX neurons could be MCH neurons, which have been shown to express AMPA and NMDA receptors (Gao et al., 2003). A method to

selectively activate a specific neuronal population within the PeFLH would clearly demonstrate the role of that population in eliciting arousal related behaviors. OX cells have been shown to exclusively express the NPY Y4 receptor subtype in the rat PeFLH and activation of this receptor on OX cells increases Fos expression (Campbell et al., 2003). By using rat pancreatic polypeptide, a selective ligand for this receptor, it should be possible to selectively activate OX neurons and more clearly demonstrate their role in eliciting LMA and ingestive behaviors.

An alternate method for selective neuronal activation is a relatively novel technique known as optogenetics, which takes advantage of the photo-sensitive properties of opsin channels to selectively activate cells expressing this photoreceptor using a beam of light (Deisseroth et al., 2006). A study in which OX neurons in the rat are targeted to genetically express channelrhodopsin-2 using a lentivirus for delivery of the gene construct has shown that, upon photostimulation of these channelrhodopsin-2 expressing OX neurons, there is a decreased latency from sleep to wakefulness (Adamantidis et al., 2007). One great advantage of this technique is that it can be employed to study any specific neuronal population within the PeFLH and their role in various arousal behaviors.

3.1.3. Refining Our View of PeFLH Neuronal Populations

The PeFLH houses neurons with a myriad of neurotransmitter phenotypes that are attributed to the various roles that this hypothalamic area plays for the regulation of arousal behavior. However, several of these neuronal populations are not confined to the PeFLH, although they are exclusive to the posterior hypothalamus. For example, OX and MCH populations are known to span mediolaterally, from the DMH right across to the

medial border of the internal capsule (Hahn, 2010). The OX population has also been demonstrated to consist of two subpopulations with different functions; the medial population is linked with general arousal and the lateral population with reward behavior (Harris et al., 2005; Harris and Aston-Jones, 2006). Anatomical studies also showed that brain areas known to be involved in the regulation of autonomic tone or behavioral arousal such as the prefrontal cortex and DMH project more heavily to the medial OX subpopulation, whereas brain areas involved with reward responses such as the ventral tegmental area and the amygdala project more heavily to the lateral OX subpopulation (Yoshida et al., 2006).

It remains uncertain whether other PeFLH neuronal candidates, like MCH neurons which share a similar but not perfectly overlapping distribution to OX neurons (Broberger et al., 1998; Hahn, 2010), can also be categorized into subpopulations that are involved with different types of behavioral responses. If the MCH population can be divided into functional subgroups, it would be interesting to determine whether the MCH and OX subgroups that elicit a particular behavioral response would have closely overlapping spatial distributions within the PeFLH, thereby creating a hypothalamic center of activation for a particular behavior. In addition, glutamatergic inputs to these MCH subpopulations would need to be investigated to find out whether, similar to afferents to OX neurons, distant brain regions would send afferent inputs preferentially to one subpopulation over another.

3.2. Concluding Remarks

Our understanding of the posterior hypothalamus has greatly expanded over the past century since the days of von Economo. Advances in experimental techniques continue to reshape our view of how specific hypothalamic areas, like the PeFLH, function to regulate various arousal related behaviors. With the discovery and characterization of an increasing number of neuronal phenotypes within the PeFLH, a finer understanding of how these populations work together to form a hypothalamic center of activation for a particular behavioral response can be established. Furthermore, a more complete understanding of the neuronal circuitry to and from this active site will help us to develop more rational therapeutic strategies to tackle abnormal behaviors when disruptions to these neuronal networks occur.

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