Behavioural and neuronal activation after microinjections of AMPA and NMDA into the perifornical lateral hypothalamus in rats

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Abbreviations:
ABC, avidin-biotin-horseradish peroxidase complex
ACSF, artificial cerebrospinal fluid
AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
CNS, central nervous system
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
PBS, phosphate-buffered saline
PeFLH, perifornical lateral hypothalamic area
TMN, tuberomammillary nucleus

ABSTRACT

The perifornical lateral hypothalamic area (PeFLH), which houses orexin/hypocretin (OX) neurons, is thought to play an important role in arousal, feeding, and locomotor activity. The present study examined behavioural effects of activating PeFLH neurons with microinjections of ionotropic glutamate receptor agonists. Three separate unilateral microinjections of either 1) AMPA (1 and 2 mM in 0.1 μl artificial cerebrospinal fluid, ACSF) and ACSF, or 2) NMDA (1 and 10 mM in 0.1 μl ACSF), and ACSF were made into the PeFLH of adult male rats. Following each injection, the rats were placed into an open field for behavioural scoring for 45 min. Rats were perfused after the third injection for immunohistochemistry for c-Fos and OX to assess the level of activation of OX neurons. Behavioural analyses showed that, as compared to ACSF conditions, AMPA injections produced a dose-dependent increase in locomotion and rearing that persisted throughout the 45 min recording period, and an increase in drinking. Injection of NMDA at 10 mM, but not 1 mM, induced a transient increase in locomotion and an increase in feeding. Histological analyses showed that while both agonists increased the number of neurons immunoreactive for c-Fos in the PeFLH, only AMPA increased the number of neurons immunoreactive for both c-Fos and OX. There were positive correlations between the number of c-Fos/OX-immunoreactive neurons and the amounts of locomotion, rearing, and drinking. These results support the role of ionotropic glutamate receptors on OX and other neurons in the PeFLH in the regulation of locomotor and ingestive behaviours.

Key Words: orexin, perifornical, glutamate, locomotion, ingestive behaviour, c-Fos.

1. Introduction

The perifornical lateral hypothalamic area (PeFLH) has been implicated in a variety of behavioural and physiological functions, including feeding [1-3], locomotion [4, 5], cardiovascular regulation [6], and sleep and wakefulness [7-9]. The PeFLH houses a heterogeneous population of neurons including those containing glutamate [10], γ-aminobutyric acid (GABA) [11], melanin-concentrating hormone (MCH) [12, 13], and orexin/hypocretin (OX) [14, 15]. 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 [16, 17]. 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 [18-20]. CNS administration of OX increases behavioural arousal [21, 22] while the absence of OX or its receptors is associated with the sleep disorder narcolepsy [23-25]. OX neurons have also been proposed to play a role in food intake. Intracerebroventricular (ICV) administration of this neuropeptide increases food intake [14, 21] and food anticipatory activity is reduced in OX knockout mice [26].

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 [27]. These

findings largely corroborate findings of the afferent connections of the PeFLH in general [28], which originate in both telencephalic [29] and brainstem nuclei [30], as well as other hypothalamic nuclei [31]. The role of these projections in modulating the activity of specific neurons within the PeFLH and physiological or behavioural 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 [31, 32].

Evidence supports the role of glutamate as an important excitatory neurotransmitter in activating OX and, likely, other neurons in the PeFLH to elicit specific behaviours. Microinjections of glutamate or agonists for specific ionotropic glutamate receptor subtypes into the LH increased feeding [33-35] and locomotor behaviours [36, 37]. Injections of glutamate into the PeFLH also promoted wakefulness while inhibiting sleep [38]. Consistent with these findings, moderate to high levels of ionotropic glutamate receptor genes and proteins [39-41], and high levels of vesicular glutamate transporter 2 immunoreactivity [42, 43] 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 neurons [44, 45] and OX neurons have been known to communicate with other OX neurons directly [46] or via a glutamate interneuron [10]. Despite these behavioural, 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 behaviours.

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 behaviours, including locomotion, feeding and drinking, by investigating the behavioural 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 behavioural 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 behavioural effects. We hypothesized that microinjections of NMDA and AMPA into the PeFLH increase arousal-related behaviours and that these increases are correlated with the increase in c-Fos immunoreactivity in OX neurons.

2. Materials and Methods

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 behavioural testing room used in the present study were maintained on a 12:12 h light:dark cycle with lights on at 7 AM 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 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 [47]). 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 behaviour-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 behavioural 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.

2.3 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 had behavioural but no neurotoxic effects when injected into the basal forebrain [48]. 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.

2.4 Behavioural experiments

2.4.1 The AMPA experiment

The behavioural 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 facilitate 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 behaviour 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 counterbalanced; no order effect was found for any of the behavioural 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 injection 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.

2.4.2 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.

2.5 Perfusion and tissue preparation

At the end of the microinjection experiments, the animals were deeply anesthetized with a ketamine/xylazine/acepromazine mixture (twice the surgical dose, i.p., see Section 2.2). 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% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). An injection cannula was inserted and remained 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).

2.6 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 [49]. 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 [49]. 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. 6).

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.

Two of the original 27 animals had an insufficient number of sections available to analyze OX neurons. Therefore, those brains were not processed for immunohistochemistry and excluded from the cell count analyses.

2.7 Behavioural analyses

Three exploratory (Locomotion, Rearing, Head Movement) and three non-exploratory (Grooming, Feeding, and Drinking) behaviours as well as Stillness were quantified from the video footage. The behavioural 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 behaviour 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 behaviours lasted 4-6 sec each in a single 10-sec bin, both behaviours were scored for that epoch (e.g., time spent in locomotion = 5 sec and time spent drinking = 5 sec).

2.7.1 Exploratory behaviours

Exploratory behaviours 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 behaviours, the *Distance Traveled* was measured using Ethovision software (ver. 3.0, Noldus, VA).

2.7.2 Non-exploratory behaviours

Three non-exploratory behaviours 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 state or asleep).

2.8 Histological Analyses

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

2.8.1 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.

2.8.2 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.

2.9 Statistical Analyses

For all behavioural data, a one-way repeated measures analysis of variance (ANOVA), followed by a *post hoc* analysis (Bonferrroni multiple comparison 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) behaviours 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 counts 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 behaviours, a Spearman's ranked correlation analysis was used. Values are expressed in mean ± standard error of the mean.

3. Results

3.1 Injection Sites

The injection sites were examined after the final (third) microinjection 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 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 behavioural effects.

3.2 Behavioural Responses

3.2.1 AMPA Injections

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 moderate levels of LMA during the first 10-15 min, in terms of both the Distance Traveled and the time spent in Locomotion, but thereafter only showed minimum levels of LMA (Fig. 3A1,2 and 3B1,2).

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 (to allow for full c-Fos expression) showed that the AMPA effects on LMA disappeared by 60-70 min after injection (data not shown).

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 (data not shown).

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 (data not shown).

3.2.1.4 Head Movement and Grooming

There were no significant changes following either concentration of AMPA compared to ACSF in the time spent in Head Movement or Grooming (see the caption of Fig. 3).

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 behaviour lasted for 50-60 min post-injection (data not shown).

3.2.2 NMDA Injections

In general, the behavioural 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 behavioural measures, the NMDA effects were clearly seen and, in some cases, significant only at the higher concentration (10 mM).

3.2.2.1 Locomotor activity

As observed in the AMPA experiment, the two measures of LMA, i.e., Distance Traveled and time spent in Locomotion, provided similar results. The total Distance Traveled during the 45 min observation period increased ($F_{2,26}$=3.88, P<0.05) following 10 mM NMDA (+184%; P < 0.05 vs. ACSF; Fig. 3A3). Likewise, the total time spent in locomotion over 45 min significantly increased ($F_{2,26}$=4.48, P<0.05) following 10 mM NMDA (+217%, P<0.05 vs ACSF; Fig. 3B3).

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 NMDA occurred mostly during the first 10-15 min after injection (Fig. 3A4 and 3B4, respectively) and this increase lasted no longer than 30 min.

3.2.2.2 Rearing, Stillness, Head Movement, and Grooming

The injection of NMDA at 10 mM resulted in a trend for an increase in the time spent in Rearing (Fig. 3C3 and 3C4) and a trend for a decrease in the time spent in Stillness (Fig. 3D3 and 3D4). As observed in the AMPA experiment, there were no significant changes following either 1 or 10 mM NMDA in the time spent in Head Movement or Grooming (see the caption of Fig. 3).

3.2.2.3 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).

3.3 c-Fos Immunoreactivity

3.3.1 Areas Surrounding the Injection Site

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. Using the protocol described in the Methods (2.8.1), the boundaries of dense c-Fos immunoreactivity were traced and overlaid for comparison between the treatment groups (Fig. 5). The mediolateral extent of the area containing dense c-Fos immunoreactivity after AMPA injections, regardless of the concentration used, was approximately 1500 μm, which was twice as wide when compared to ACSF injections. After NMDA injections, regardless of the concentration used, the area of dense c-Fos immunoreactivity was slightly smaller when compared to AMPA injections, with the width of approximately 1100 μm.

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. 6), 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 1), 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 1), and the analyses of this measure revealed several significant differences between treatment groups as described below.

3.3.2.1 AMPA Injections

As shown in Table 1, 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 and 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.

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 1).

3.4 Correlations Between c-Fos+/OX+ Labeling and Behavioural Measures

To determine whether the c-Fos immunoreactivity of OX neurons had any role in the behavioural 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 behavioural activation that showed a significant increase following NMDA or AMPA treatments, including: Distance Traveled, times spent in LMA, Rearing, and Drinking, and the amount of 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. 7A), and time spent in Locomotion (ρ =0.53; P<0.01; n=25; Fig. 7B), Rearing (ρ =0.45; P<0.05; n=25; Fig. 7C), and Drinking (ρ =0.56; P<0.01; n=25; Fig. 7D). There was no significant correlation between the percentage of c-Fos+/OX+ cells and Feeding (ρ =0.147; P=0.47). Overall, AMPA produced a higher level of behavioural activity along with more activation of OX neurons compared to NMDA.

4. Discussion

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

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 behavioural responses under multiple conditions, repeated injections into the same brain region could affect the viability of neurons around the injection site and, consequently, behavioural 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 behavioural responses under any of the drug conditions. The c-Fos response of surrounding neurons to microinjections of AMPA and NMDA is consistent with this interpretation.

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 45 min 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 those reported in studies that used food preparations (e.g., milk-mash) that provide more accurate measures of consumption in order to study the effects of injections of OX or glutamate and its agonists into the PeFLH in food-satiated rats [2, 50].

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.

Finally, we noted that the percentage of c-Fos positive OX neurons after ACSF injection was higher in the AMPA injected group than in the NMDA injected group. We have no obvious explanation for this discrepancy. We cannot exclude the possibility that immunohistochemical sensitivity for either c-Fos or OX was different between the AMPA and NMDA experiments, which were completed at different time points.

4.2 Behavioural 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 behaviours, 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 [5, 51, 52] or administration of GABA receptor antagonists or glutamate into the PeFLH produced a broad range of LMA, from increased general locomotion to flight-like locomotor responses [37, 53].

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 [34, 35, 54]. 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 behaviour 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 [3, 55], whereas electrical stimulation of the PeFLH immediately lateral to the dorsomedial hypothalamic nucleus (DMH) or the ventromedial hypothalamic nucleus resulted in dipsogenesis [56, 57]. In addition, a previous study has shown that administration of glutamate or glutamate receptor agonists into the lateral PeFLH did not initiate water consumption [34]. 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 inputs are local glutamate interneurons within the PeFLH , which have been shown to serve as an intermediary for communication from an OX neuron to another OX neuron [10], and putative glutamatergic neurons in the DMH, a nucleus implicated in the regulation of circadian rhythms and wake related behaviours such as feeding and locomotor activity [31]. Extrahypothalamic sources of glutamate afferents to the PeFLH include the ascending projections from the lateral parabrachial nucleus, involved in visceral and other sensory processing [32], and reticular neurons along the brainstem [58, 59], which are involved in wakefulness and related behaviours. The PeFLH also receives descending glutamatergic projections from the basal forebrain [42] and the medial prefrontal cortex [60]. While some of these glutamatergic afferents have been demonstrated to project directly to OX cells [27, 32], 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 behaviours will serve to further our understanding of glutamatergic regulation of arousal-related behaviours in this region.

4.3 Role of OX Neurons in Behavioural 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 behaviours when both drug treatment groups were combined. These results suggest that OX neurons could have been involved in eliciting these behavioural responses to AMPA and NMDA injections.

The positive correlations between the number of c-Fos+/OX+ cells and exploratory behaviours is consistent with previous findings that increased locomotion was accompanied by increased discharge in OX neurons [18] and increased OX levels in the brain [61] and cerebrospinal fluid [62]. OX neurons also increased c-Fos expression during active wake, but not during quiet wake [63]. OX neurons may increase LMA through their projections to many nuclei of the wake system, including the TMN and LC [17]. Further support is provided by findings that OX or OX neurons activated histaminergic neurons in the TMN [64] and noradrenergic neurons in the LC [65], and that administration of OX directly into the LC induced hyper-locomotion and rearing activity in rats [21].

We also found a positive correlation between the number of OX neurons expressing c-Fos and drinking behaviour. This is consistent with an increased water intake after central administration of OX [66]. Anatomically, the PeFLH receives a direct input from the subfornical organ, a well-documented site for osmotic regulation [67], which is also reciprocally innervated by OX neurons [68]. 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 behaviour. For example, the number of OX immunoreactive cells in the PeFLH is known to be increased during fasting [26], feeding is increased after intracerebroventricular administration of OX [14, 69, 70], and OX-induced feeding requires activation of the NMDA receptor [50]. However, as mentioned above, feeding responses to glutamate and its receptor agonists were strongest when the drugs were administered into the lateral PeFLH [54]. Furthermore, OX neurons that expressed c-Fos after feeding following food deprivation were located primarily in this area [71]. 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 behavioural 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 behavioural effects as observed after AMPA injections. In fact, the brief behavioural 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 [72]; 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 behaviours, it is likely that non-OX neurons in the PeFLH were also recruited to elicit these behaviours, 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 [13, 73], glutamate interneurons [10], 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 [74] but different for locomotion and sleep-wake regulation. MCH has been associated with inhibition of locomotor responses [75] and implicated in the regulation of rapid eye movement sleep [76]. Experiments involving selective activation of either of these two distinct populations would help determine the specific roles of these cell populations in the observed behavioural responses.

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 behaviours 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 behaviours and support the role of glutamatergic regulation of OX cells within the PeFLH on locomotive and ingestive behaviours.

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Table 1. 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 in each animal and the number of animals examined in each condition is indicated in parentheses. *p<0.05, ipsilateral versus contralateral side.

FIGURE CAPTIONS

Figure 1. 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.

Figure 2. 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 black and white circles (middle column) and black squares (right column) indicate the estimated locations of injection sites for AMPA (n=13) and NMDA (n=14), respectively, based on the last injection in each animal. The two white circles for the AMPA experiment indicate the injection sites of two animals (out of 13) that were not included in imunohistological examination (see the Materials and Methods).

Figure 3. 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, 2 mM AMPA, 1 mM NMDA, or 10 mM NMDA. Values are presented as a total (AMPA, Coulmn 1; NMDA, Coumn 3) and as a time course (AMPA, Column 2; NMDA, Coulumn 4). For each condition, n=13 for AMPA treated animals and n=14 for NMDA treated animals. Each rat received a total of three injections with either ACSF and low and high concentrations of AMPA, or ACSF and low and high concentrations of NMDA in a random order. * p<0.05 versus ACSF; ** p<0.01 versus ACSF; +p<0.05 versus 1mM NMDA. In addition to these behavioural measures, Head Movement and Grooming were analyzed. No significant changes were seen following either concentration of AMPA compared to ACSF for the time spent in Head Movement (217 ± 34, 319 ± 52, 323 ± 46, for ACSF, 1 mM, and 2 mM AMPA, respectively) or Grooming (173 ± 32, 136 ± 30, 100 ± 26, for ACSF, 1 mM, and 2 mM AMPA, respectively). Similarly, no significant changes were seen following either concentration of NMDA compared to ACSF for the time spent in Head Movement (359 ± 35, 396 ± 34, 360 ± 27, for ACSF, 1 mM, and 10 mM, respectively) or Grooming (194 ± 21, 217 ± 21, 167 ± 32, for ACSF, 1 mM, and 10 mM, respectively).

Figure 4. 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.

Figure 5. 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 Materials and Methods for details).

Figure 6. 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+.

Figure 7. Relationships between OX+/Fos+ cell counts and Distance Traveled (A), time spent in Locomotion (B), time spent in Rearing (C), and time spent in Drinking (D) during 45 min period after microinjections of ACSF (diamond), 1 mM AMPA (white square), 2 mM AMPA (black square), 1 mM NMDA (white circle), and 10 mM NMDA (black circle). Correlation coefficients were obtained using a Spearman ranked correlation analysis. For each behavioural measure, n = 25. Moderate but significant correlations are seen between the OX+/Fos+ cell counts and all 4 behavioural measures.

Table 1. 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.

		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

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.

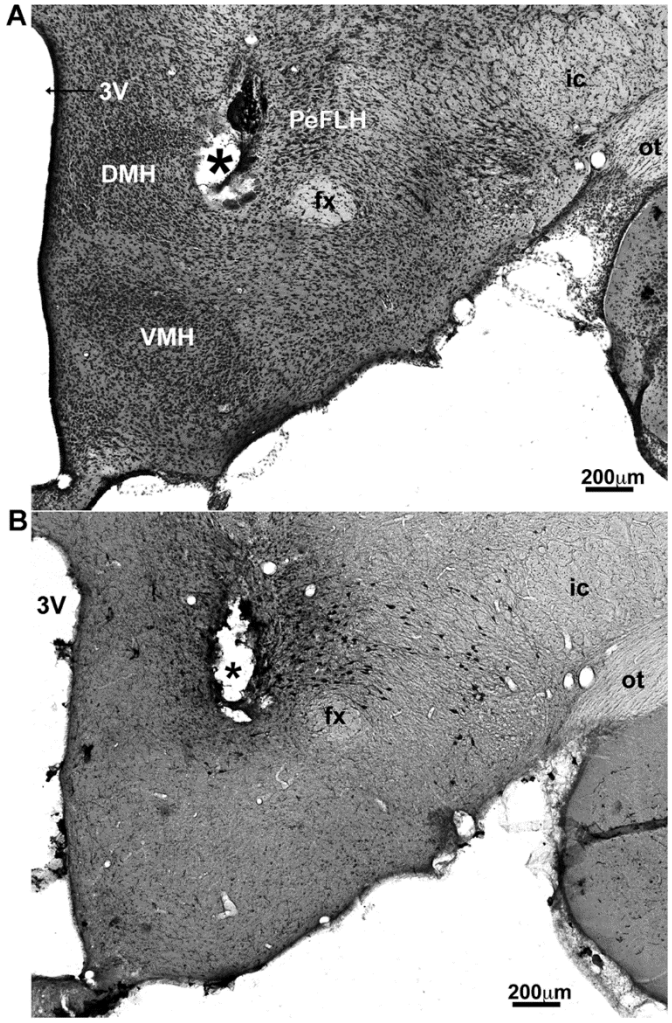

Figure 1

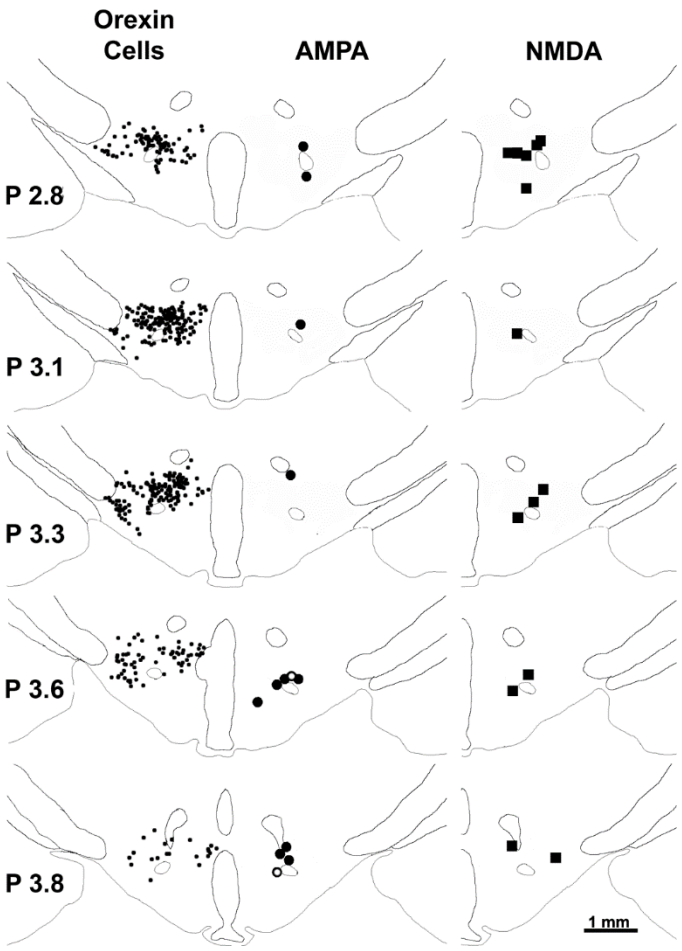

Figure 2

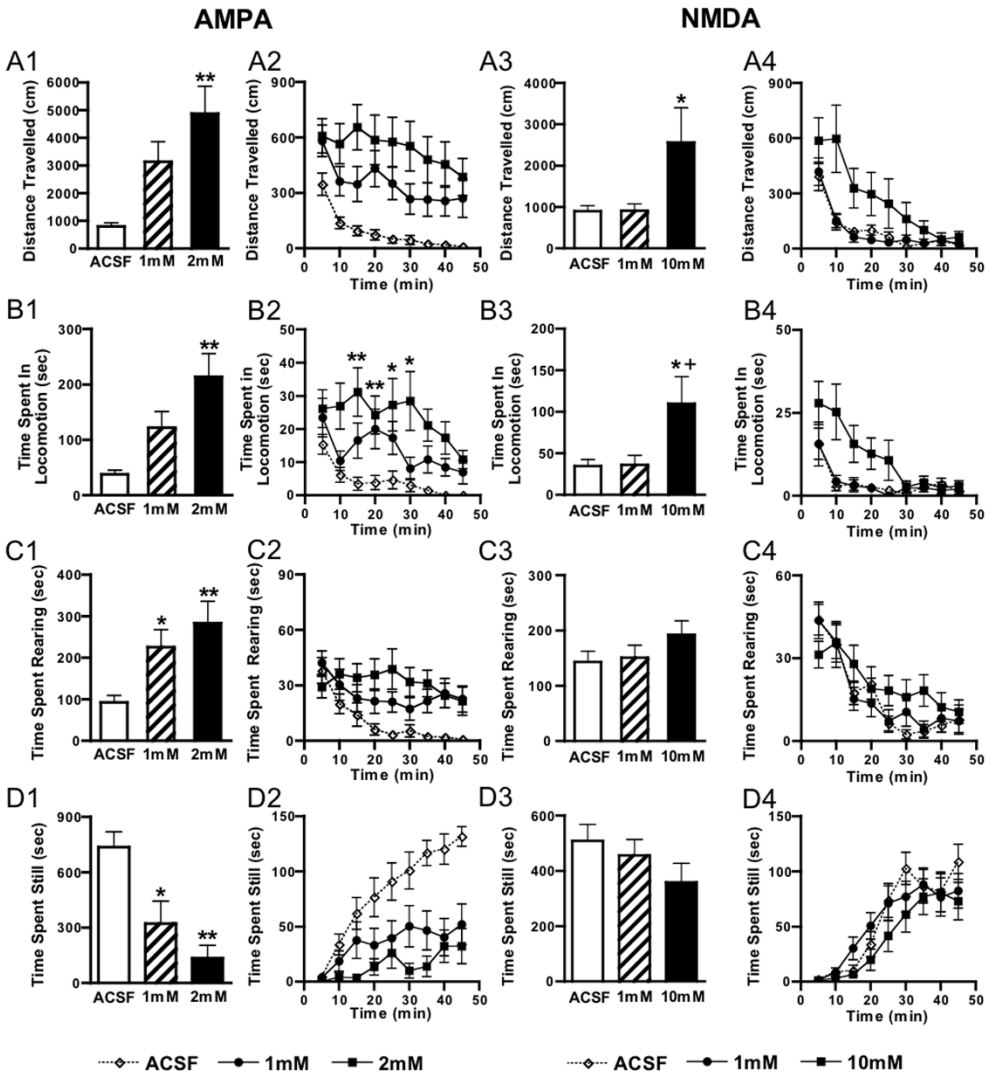
AMPA
NMDA
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
Distance Travelled (cm)
Time Spent In Locomotion (sec)
Time Spent in Locomotion (sec)
Time Spent Rearing (sec)
Time Spent Still (sec)
ACSF 1mM 2mM
ACSF 1mM 10mM
Time (min)
ACSF
1mM
2mM
10mM

Figure 3

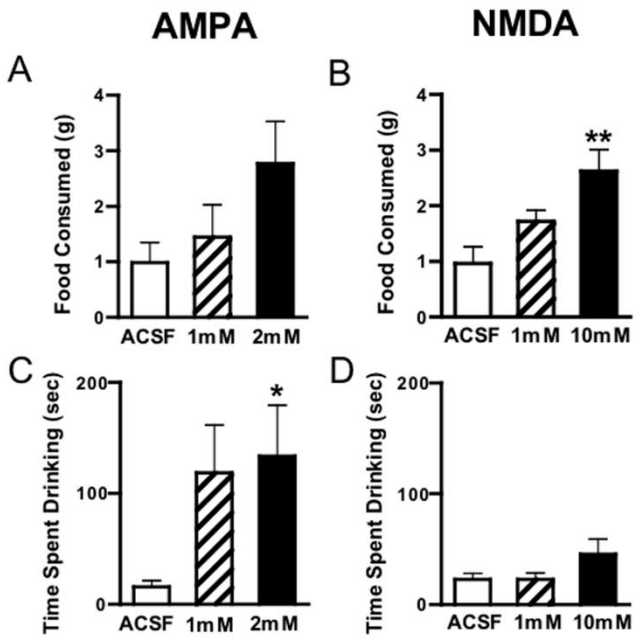
AMPA
NMDA
A
B
C
D
Food Consumed (g)
Time Spent Drinking (sec)
ACSF
1mM
2mM
10mM
*
**

Figure 4

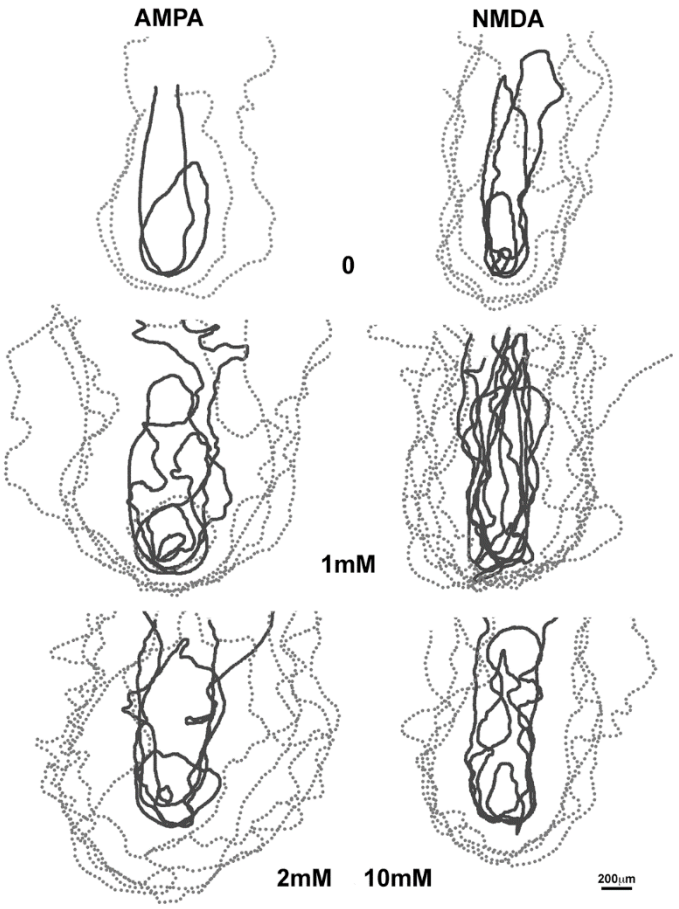

Figure 5

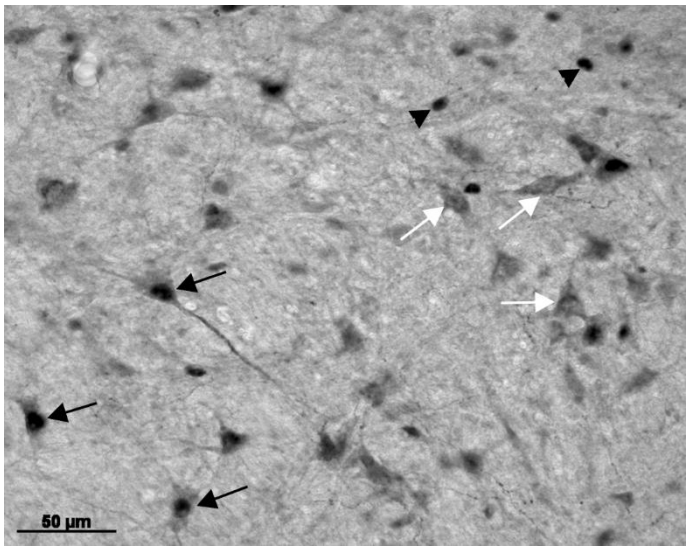

Figure 6

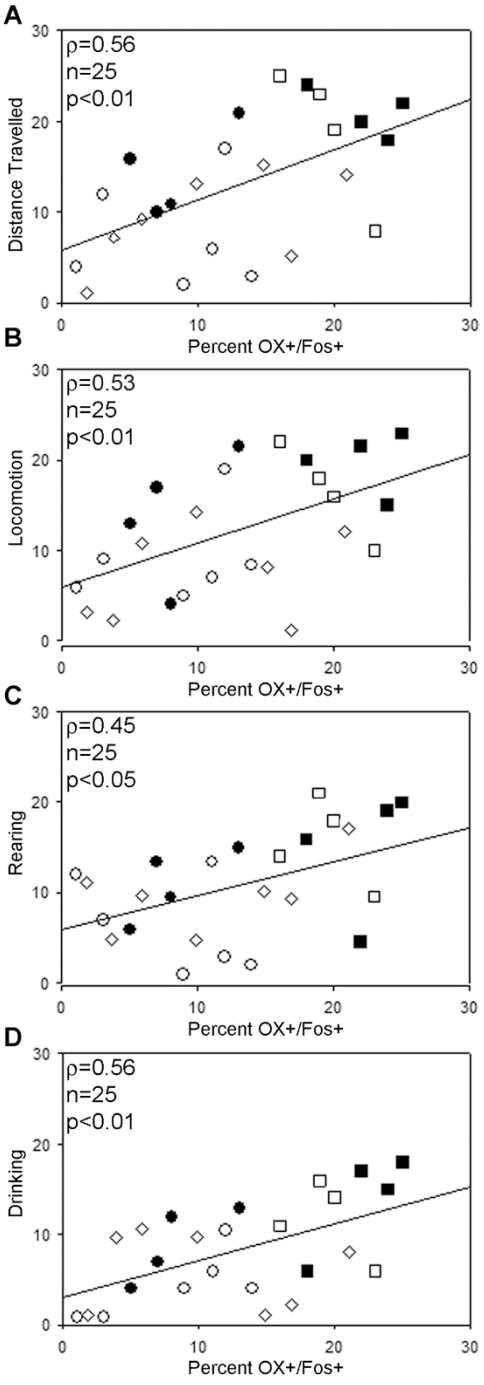
A
ρ=0.56
n=25
p<0.01
Distance Travelled
Percent OX+/Fos+
B
ρ=0.53
n=25
p<0.01
Locomotion
Percent OX+/Fos+
C
ρ=0.45
n=25
p<0.05
Rearing
Percent OX+/Fos+
D
ρ=0.56
n=25
p<0.01
Drinking
Percent OX+/Fos+

Figure 7