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**EXCITATORY AND INHIBITORY NEURAL CONTROL OF  
SEXUAL MATURATION IN THE FEMALE RAT**

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Submitted in Partial Fulfillment of the Requirements for the  
Degree of Doctor of Philosophy  
at Dalhousie University  
July 1990



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*This thesis, is dedicated to my parents, Shirley Wilma MacDonald  
and Dr. Angus Carleton MacDonald*

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## Abstract

This Thesis examines both the excitatory and inhibitory neural mechanisms which regulate sexual maturation in the female rat. Single daily prepubertal injections of the excitatory glutamate agonist N-methyl-D-aspartate (NMDA) significantly advanced the day of first ovulation (vaginal opening; V.O.) whereas similar treatment with the NMDA antagonists MK-801 or dextrorphan effectively delayed but did not prevent V.O.. NMDA readily stimulated LH secretion in prepubertal rats, an effect which could be blocked by MK-801. NMDA-induced hypothalamic cellular activity was immunocytochemically localized through expression of the early-response gene (proto-oncogene) c-fos and was also prevented by pre-injection of MK-801. Neonatal administration of monosodium glutamate (MSG), known to destroy glutamatergic neurons in the arcuate nucleus (ARC), significantly advanced the time of V.O., possibly by the removal of inhibitory  $\beta$ -endorphin neurons. These results suggest that the excitatory neurotransmitter glutamate may be important in the control of GnRH/LH release and the process of sexual maturation.

The inhibitory opiate regulation of puberty was examined using two opiate agonists, morphine and fentanyl citrate. When these were administered orally, via the drinking water, or by subcutaneous osmotic minipumps, V.O. was significantly delayed but not prevented. This suggests that the hypothalamic opioid system can be desensitized to allow V.O. to occur even in the face of continuous agonist treatment. However, a combination of fentanyl and MK-801 considerably extends the delay in maturation. This suggests the presence of distinct but converging excitatory and inhibitory pathways. Finally, *in vitro* micropunch studies demonstrated that the mu agonists DAGO and fentanyl can decrease hypothalamic cell surface mu-opioid receptor number without affecting binding affinity. This is a simple but effective technique with which to examine opioid receptor regulation.

These studies provide convincing evidence that excitatory amino acids and endogenous opioid peptides play a critical role in the neural control of sexual maturation in the female rat.

## List of Abbreviations

AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
Ant. Pit.	anterior pituitary
APV	amino-5-phosphonovalerate
ARCN	arcuate nucleus
$\beta$ -END	beta endorphin
BBB	blood brain barrier
$B_{max}$	maximal specific binding
BW	body weight
CL	corpus luteum
Ci	Curie
$^{\circ}\text{C}$	degrees Celcius
d	day
DA	dopamine
DAB	diaminobenzidine
DAGO	([D-Ala <sup>2</sup> ,N-Me-Phe <sup>4</sup> , glyol <sup>5</sup> ] enkephalin
DNQX	6,7-dinitro-quinoxaline-2,3 dione
DPBS	Dulbecco's phosphate buffered saline
DPBS+	(DPBS; HEPES (10mM); glucose (0.1%); H <sub>2</sub> O <sub>2</sub> (0.004%)
EAA	excitatory amino acid
EB	estradiol benzoate
ED <sub>50</sub>	dose required to produce half maximal stimulation
EOP	endogenous opioid peptides

E <sub>2</sub>	estradiol
Fig.	figure
fmoles	femtomoles
FSH	follicle-stimulating hormone
g	gram
GH	growth hormone
GnRH	gonadotropin-releasing hormone
[ <sup>3</sup> H]	tritium
hr	hour
i.p.	intraperitoneally
i.u.	international units
i.v.	intravenously
KA	kainate
K <sub>D</sub>	equilibrium dissociation constant
kg	kilogram
L	liter
LH	luteinizing hormone
MBH	mediobasal hypothalamus
μg	microgram
μl	microliter
μM	micromolar
mg	milligram
min	minute
ml	milliliter
M	molar
MK-801	dibenzocyclohepteneimine
mM	millimolar

MPO	medial preoptic area
MSG	monosodium glutamate
NAL	naloxone
ng	nanogram
nM	nanomolar
NMA	N-methyl-D,L-aspartate
NMDA	N-methyl-D-aspartate
NRS	normal rabbit serum
QA	quisqualate
NSB	non-specific binding
OVX	ovariectomy
p.	page
PB	phosphate buffer
PBS	phosphate buffered saline
PCP	phencyclidine
PKC	protein kinase C
PMSG	pregnant mare serum gonadotropin
POMC	proopiomelanocortin
PRL	prolactin
s.c.	subcutaneously
s.e.m.	standard error of the mean
SA	specific activity
SB	specific binding
SCN	suprachiasmatic nucleus
SD	Sprague Dawley
SFO	subfornical organ
TC	total count

Tris \_\_\_\_\_ tris (hydroxymethyl) aminomethane  
U14,624 \_\_\_\_\_ 1-phenyl-3-(2-thiazolyl)-2-thiourea  
V.O. \_\_\_\_\_ vaginal opening  
WT \_\_\_\_\_ weight

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## Section I. GLUTAMATE AND SEXUAL MATURATION

### A. Neural Control of Sexual Maturation

A precise description of the neural mechanisms responsible for sexual maturation remains elusive. (Ojeda et al. 1986a; Ojeda and Urbanski 1988; Foster et al. 1986). It is generally agreed that the one compulsory event in this process is the appearance of pulsatile gonadotropin-releasing hormone (GnRH) secretion thought to be a result of a neural timing mechanism, the so-called GnRH pulse generator. This then provides the stimulus for secretion of anterior pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) in sufficient quantities to initiate the production of gonadal steroids. A number of theories have been proposed to account for the process of sexual maturation, the most popular being the "gonadostat" hypothesis (Hohlweg and Junkmann 1932; Ramirez and McCann 1963). It states that as an animal matures there is a decrease in the sensitivity of the hypothalamic-pituitary unit to steroid negative feedback; this shift in sensitivity can thus be interpreted as the removal of an inhibition. This implies that during infancy, steroids actively suppress gonadotropin secretion via negative feedback, but with increasing maturity more steroid is required to maintain this suppression. This inhibitory shift (the 'resetting of the gonadostat') allows LH secretion to rise. Studies by Steele and Weisz (1974) have shown that much smaller amounts of estradiol (E<sub>2</sub>) suppress the post castration rise of gonadotropins of prepubertal than of pubertal or adult rats. Similar conclusions have

been reached from experiments on female lambs (Foster et al. 1986). That this "resetting" occurs at the neural level and is not just a result of age-related metabolic degradation of injected E<sub>2</sub> has been demonstrated by Docke et al. (1978). This group, using intrahypothalamic E<sub>2</sub> implants, demonstrated that at the time of puberty the effectiveness of E<sub>2</sub> to suppress gonadotropins is decreased.

The gonadostat hypothesis has been further refined and expanded as a broader understanding of hypothalamic control of gonadotropin release has emerged. There appears to be substantial evidence suggesting that an endogenous opioid system exerts a tonic inhibitory control on the immature hypothalamic-pituitary-LH axis (for a review see Wilkinson and Landymore 1989). The age-related removal of this opioid inhibition, or decrease in sensitivity to opioids, takes place in parallel with the decrease in steroid sensitivity as sexual maturation proceeds, and therefore may represent the specific neurochemical mechanism for the resetting of the gonadostat (Bhanot and Wilkinson 1983).

An alternative explanation for the neural control of sexual maturation involves the influence of possible excitatory mechanisms (Ruf 1982; Wray and Hoffman 1986a). In this theory, puberty is coincident with a completed excitatory circuitry (synaptogenesis) which provides the drive for increased pubertal secretion of GnRH. Thus, sexual maturation may be the result of a maturational increase in the stimulation of the hypothalamic areas governing GnRH secretion. This is clearly the antithesis of the removal of a hypothalamic restraint. Experiments performed by Matsumoto and

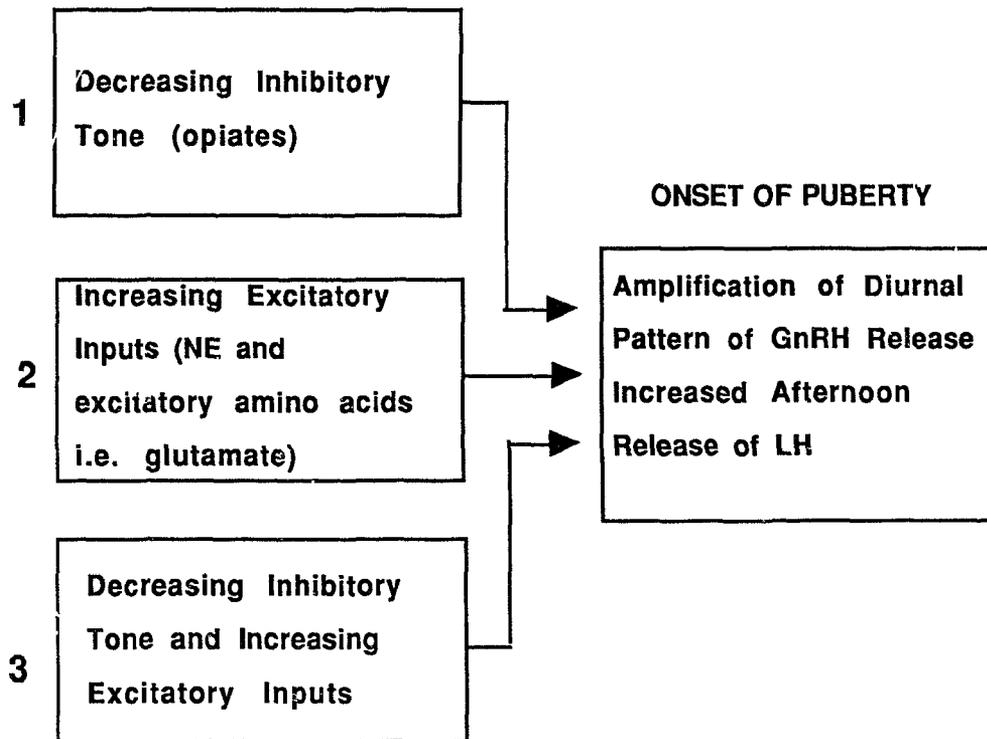
Arai (1977), using transmission electron microscopy, support the synaptogenesis hypothesis. They demonstrated that accelerated synaptogenesis in the arcuate nucleus (ARCN) occurred concurrently with precocious ovulation in pregnant mare serum gonadotropin (PMSG)-treated female rats. These neural connections are mostly axodendritic, and the ARCN, which is part of the mediobasal hypothalamus (MBH), is of central importance to the onset of puberty and the regulation of cyclic hormone secretion (Ruf 1982). Arai et al. (1986) have also provided evidence that synaptogenesis is stimulated by E<sub>2</sub> and that this action is limited to the prepubertal brain of intact female rats. More recent studies by Rodriguez-Sierra and Clough (1987) demonstrate that the acute E<sub>2</sub>-mediated increase in synaptogenesis in the ARCN occurs concurrently with the premature activation of phasic pituitary gland LH secretion. However, the neurochemical nature of these new synapses remains unknown.

An important event that appears to signal the onset of puberty is the development of a diurnal rhythm of pulsatile LH secretion (Beck and Wuttke 1980; Kelch et al. 1983). Classical work by Boyar (1978) shows that pulsatile LH levels increase during sleep and decrease during the waking hours in prepubertal children. When sexual maturation has been reached, the diurnal variation of LH secretion but not the pulses, disappears. Remarkably, a similar diurnal pattern of LH secretion occurs in both peripubertal monkey (Teresawa et al. 1984; Plant 1988) and rat (Ojeda and Urbanski 1988). In the female rat, Urbanski and Ojeda (1985) have shown that throughout the juvenile period (Day 20-30 of life) neither the frequency or amplitude of LH pulses changes significantly. In contrast, during the

early peripubertal period (Day 30 to first ovulation) there is a dramatic increase in both LH pulse amplitude and mean plasma LH levels that is only seen in the afternoon. The establishment of this diurnal rhythm is thought to be a centrally driven, gonadal-independent event (Ojeda and Urbanski 1988) perhaps indicating the synchronization of GnRH impulses resulting from synaptogenesis in the hypothalamic neural circuitry. As puberty approaches, afternoon increases in LH secretion stimulate the ovaries to produce more E<sub>2</sub> resulting in LH minisurges (Urbanski and Ojeda 1986). These minisurges result in further ovarian stimulation and E<sub>2</sub> production that culminates in the proestrous LH surge and first ovulation (approximately Day 35).

Bhanot and Wilkinson (1983) have suggested a possible conciliation of the gonadostat and synaptogenic theories. In its simplest form, the gradual decrement in opiate inhibition, or the resetting of the hypothetical gonadostat, can be accounted for by a concomitant activation of excitatory inputs (See Fig. 1). A more precise definition of this excitatory drive will be discussed later in this literature review.

Although numerous studies have implicated the catecholaminergic system as the predominant source of excitatory input mediating GnRH release (Barraclough and Wise 1982; Ramirez et al. 1984; Kalra 1986), recent developments in neuroendocrine research suggest that a number of other neurotransmitters may also be significant constituents of this stimulatory process (Ojeda and Urbanski 1988). The excitatory amino acid (EAA) L-glutamate and its activity at the



**Fig. 1.** Possible mechanisms underlying the onset of puberty in the female rat. Available evidence suggests that the simultaneous occurrence of events (1) and (2) (shown as (3)) may be the underlying mechanisms responsible for the development of the afternoon increase in LH release which signals the initiation of puberty. (From Ojeda and Urbanski 1988)

N-methyl-D-aspartate (NMDA) receptor has been the subject of extensive research, especially in the area of neural plasticity and synaptogenesis (Cotman et al. 1988). Recent studies have implicated NMDA receptors as an essential part of the cellular mechanism underlying certain forms of long term potentiation (LTP) (Collingridge and Bliss 1987). In several hippocampal pathways activation of NMDA receptors is required for the induction of LTP, whereas antagonism of these receptors prevents its effects.

The studies described in this thesis have examined both the inhibitory and excitatory influences mediating the control of the onset of puberty in the female rat. Experiments on the inhibitory control, focus on the effects of exogenous opiates (morphine and fentanyl citrate) on the timing of sexual maturation. The effects of opiates and opioid peptides on hypothalamic mu-opioid receptor regulation *in vitro* are also examined. The excitatory mechanisms that may control the onset of puberty are investigated by examining the effects of NMDA receptor activation and inhibition on the timing of puberty. The effects of excitatory amino acids at the cellular level have also been assessed.

The remainder of this Introduction provides a background summary of glutamate as an excitatory neurotransmitter; the glutamate receptors, and the role of glutamate as a possible hypothalamic regulator of sexual maturation.

## B. Glutamate as a Neurotransmitter

During the last 50 years the role of glutamate in the brain has been the subject of numerous studies, and several functions have been ascribed to it. Krebs (1935) provided preliminary evidence that glutamate is metabolically active in the CNS and suggested that it may play a role in intermediary metabolism. Others (Weil-Malherbe 1950) have investigated glutamate function in the detoxification of ammonia in the brain. Early clinical studies suggested that glutamate might be beneficial in a number of neurological disorders including hypoglycemic coma, epilepsy and mental retardation (Weil-Malherbe 1950; Waelsch 1951). Other distinctive properties of glutamate revealed during this period included its unusual metabolic compartmentation (Berl et al. 1961); the potent excitatory action on vertebrate CNS neurons (Curtis et al. 1959); its ability to initiate cell death in the retina (Lucas and Newhouse 1957); and its importance as the immediate metabolic precursor of gamma-aminobutyric acid (GABA) (Roberts and Frankel 1950). More recent studies have shown that glutamate has an exceptionally low rate of penetration across the blood-brain-barrier (Oldendorf 1971); is found in low concentrations in the cerebral spinal fluid (Gjessing et al. 1972); and, in its monosodium form, has been implicated as a causal factor in the Chinese restaurant syndrome (Schaumberg et al. 1969). Although only a decade ago the role of glutamate in the mammalian central nervous system was considered controversial, it has now been established as the major excitatory neurotransmitter in the CNS and thus satisfies the main criteria for classification as a

neurotransmitter. These criteria include (1) presynaptic biosynthesis and localization in specific nerve terminals; (2) specific release by physiological stimuli in concentrations great enough to evoke a postsynaptic response; (3) identity of action (i.e., postsynaptic actions of endogenous transmitter are mimicked by exogenous substance); (4) existence of neuronal mechanisms, such as reuptake and metabolism, that terminate neurotransmitter action (Fagg and Foster 1983) and (5) the presence of specific receptor sites (see next section).

## C. The Glutamate Receptors

The number of receptors that bind glutamate consists of at least five different subtypes (Monaghan et al. 1989). These five classes are activated preferentially by (1) N-methyl-D-aspartate (NMDA), (2) quisqualate (QA), (3) kainate (KA), (4) L-2-amino-4-phosphonobutyric acid (L-AP4), and (5) *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) (Monaghan et al. 1989).

### 1. *The N-methyl-D-aspartate (NMDA) Receptor*

The NMDA receptor is by far the best characterized of all glutamate receptor subtypes in the CNS (Mayer and Westbrook 1987). The prototype agonist for this receptor is NMDA but recent evidence (Garthwaite 1985) suggests that the relatively high potency of this substance in intact neural tissue results from its low rate of transport from the extracellular fluid i.e. reduced uptake. In fact radioligand binding experiments in broken cell preparations performed by Olverman et al. (1984) and others (Fagg and Matus 1984) have demonstrated that L-glutamate displays an affinity for the NMDA receptor that is 10-fold greater than NMDA itself, whereas L-aspartate, while binding preferentially to the NMDA site, is 10 times less potent than L-glutamate.

The development of competitive NMDA antagonists (bind directly to the glutamate/NMDA receptor) such as 2-amino-5-phosphonovalerate (AP5 or APV) (Davies et al. 1981; Davies and Watkins 1982) D-2-amino-7-heptanoate (AP7) (Evans et al. 1982) and 3-((±) 2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP)

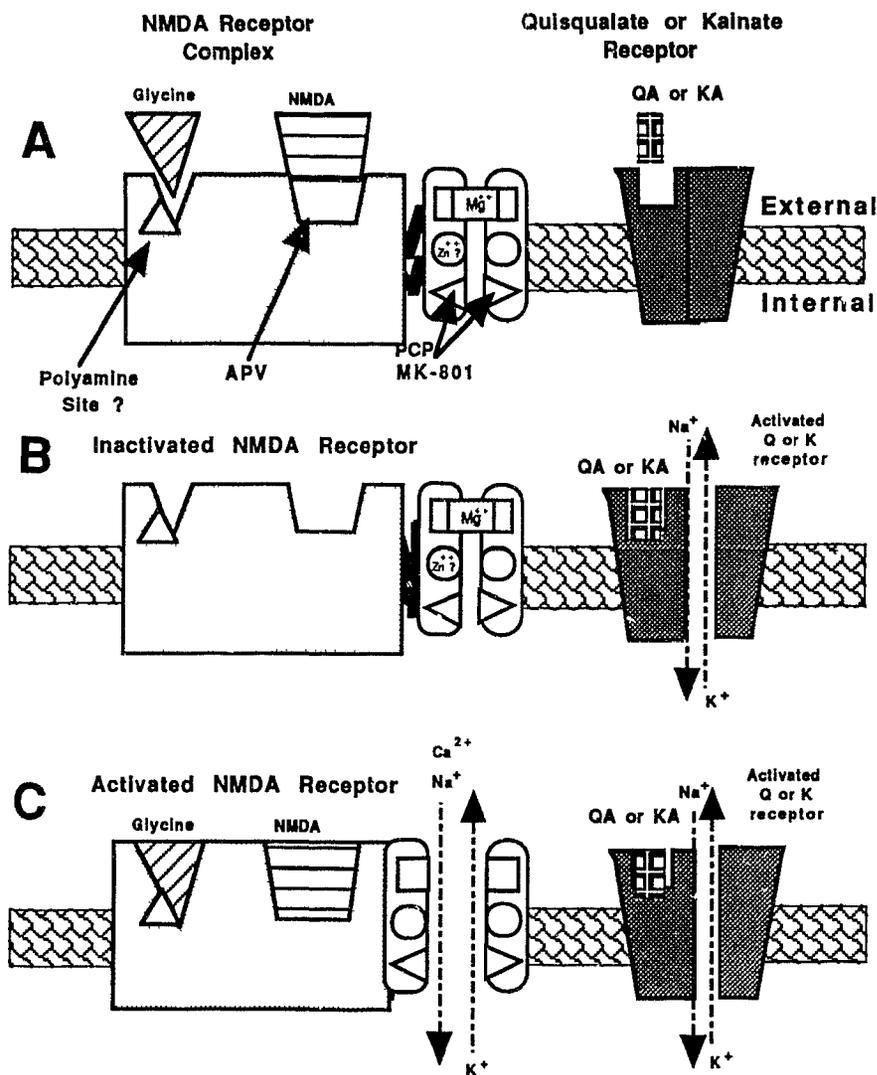
(Lehman et al. 1988) have contributed immensely in determining the anatomical distribution of NMDA receptors (Jarvis et al. 1987). Autoradiographical studies using the [ $^3\text{H}$ ] form of these compounds (Monaghan et al. 1984b; Monaghan et al. 1987; Maragos et al. 1986) have shown that NMDA sites in the brain are predominantly located in cortical structures, basal ganglia, and sensory associated systems, the greatest levels being found in the hippocampal CA1 termination of the Schaffer collateral pathway. NMDA receptors and other glutamate receptor subtypes are also found throughout the hypothalamus (Greenamyre et al. 1984; Cotman et al. 1987) and will be discussed later in section D on Glutamate and Sexual Maturation.

Preliminary evidence for an NMDA noncompetitive antagonist (binds to a site other than the glutamate/NMDA receptor) was demonstrated by Lodge and coworkers (1982) who found that phencyclidine (PCP) and ketamine reduced the excitatory action of NMDA on cat spinal neurons. Electrophysiological (Martin and Lodge 1985) and radioligand binding assays (Harrison and Simmonds 1985) using combinations of ketamine and the competitive antagonist AP5 indicated that these two compounds do not share the same site of action. Subsequent studies (Lodge et al. 1986) suggested that ketamine and related compounds prevented the glutamate or NMDA induced influx of extracellular  $\text{Ca}^{2+}$  by interacting with the NMDA receptor-associated channel. Other compounds that correlate well with PCP-like effects in binding, neurophysiological and behavioral studies include the benzomorphans or sigma opiates: SKF 10047 (Martin 1981); dextrorphan, levorphanol and dextromethorphan (Church et al. 1985; Mendelsohn et al. 1984); and more recently MK-

801 (Kemp et al. 1986), considered to be the most potent antagonist within this class (Wong et al. 1986).

In contrast to fast-acting, voltage-independent synaptic transmission mediated by an increase in  $\text{Na}^+$  and  $\text{K}^+$  permeability, the conductance that is activated by NMDA receptor agonists is voltage dependent (Engberg et al. 1979). This voltage-dependence is due to a block by extra-cellular  $\text{Mg}^{2+}$  (Nowak et al. 1984) and only occurs at potentials more negative than  $-20\text{mV}$ . As the membrane is depolarized, most likely by activation of voltage-independent QA and/or KA receptors, the channel block by  $\text{Mg}^{2+}$  is removed and the resultant inward current further amplifies the depolarization. Cation channels regulated by the NMDA receptor are permeable to  $\text{Ca}^{2+}$  as well as  $\text{Na}^+$  and  $\text{K}^+$ . Increases in intracellular  $\text{Ca}^{2+}$  have been shown to occur after application of NMDA agonists, using intracellular measurements with ion sensitive electrodes (Pumain and Heineman, 1985) and  $\text{Ca}^{2+}$  sensitive dyes (Murphy et al. 1987).

Recent evidence suggests that the NMDA-receptor-ionophore complex consists of multiple components that alter the activity of the receptor, and is therefore analogous to the GABA-benzodiazapine receptor complex (Kleckner and Dingledine, 1988). The NMDA receptor complex (See Fig. 2) has binding sites for (1) a transmitter such as glutamate (2) an allosteric potentiator, glycine (3) PCP-like compounds, most likely within the receptor gated channel (4)  $\text{Mg}^{2+}$ , within the receptor gated channel which binds in a voltage-dependent manner (5)  $\text{Zn}^{2+}$ , an inhibitory divalent cation that is an effective antagonist comparable to  $\text{Mg}^{2+}$  but does not bind in a



**Fig. 2.** (A) Schematic representation of the NMDA receptor complex and its association with the kainate (KA) or quisqualate (QA) receptor. Depicted are the sites of competitive (APV) and non-competitive (PCP, MK-801) NMDA antagonist action. The location of the polyamine site is controversial. (B) The NMDA receptor in its inactivated state. Note the activation of the QA or KA still occurs and most likely aids in the depolarization required to remove the  $Mg^{2+}$  blockade of the NMDA ion channel. (C) When NMDA and its allosteric regulator, glycine bind to the receptor complex,  $Na^{+}$  and  $K^{+}$  conductance within the NMDA ion channel increases. The cell becomes more depolarized (-20 mV), the  $Mg^{2+}$  block is removed, and  $Ca^{2+}$  enters the cell. The influx of  $Ca^{2+}$  results in a variety of neuronal responses.

voltage-dependent manner (Peters et al. 1987) and (6) polyamines (i.e. spermine and spermidine) which, in low concentrations, appear to enhance [<sup>3</sup>H]MK-801 binding. Although the exact location of this site is highly controversial, the glycine site and the voltage gated channel appear to be two possible binding sites for these compounds. (Ransom and Stec 1988; Williams et al. 1989).

NMDA and other EAA can activate the phosphoinositol and cyclic nucleotide second messenger systems (see also section 2 on the Quisqualate and Kainate receptors below). NMDA application to cultured cerebellar granule cells (Nicoletti et al. 1986c) stimulates inositol phospholipid (IP) metabolism as well as cyclic GMP production (Novelli et al. 1987). Interestingly the magnitude of both glutamate-induced IP metabolism (Nicoletti et al. 1986a) and cyclic GMP formation (Garthwaite and Balazs 1978; Foster and Roberts 1983) exhibit large declines during development.

## 2. *Quisqualate (QA) and Kainate (KA) Receptors*

The proposal that QA and KA act at separate receptors was based initially on the use of the antagonist L-glutamic acid diethyl ester (GDEE) (Haldeman and McLennan 1972; McLennan and Liu 1982). Radioligand assays have identified QA and KA preferring sites with distinct agonist selectivities (Slevin et al. 1983; Murphy et al. 1987; Olsen et al. 1987). Domoate and KA demonstrate the most potency at the KA receptor (Davies et al. 1979; Slevin et al. 1983) whereas DL-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and QA are most potent at the QA binding site (Olsen et al. 1987). Autoradiographical studies (Monaghan et al. 1984a) have shown

different anatomical distributions of these sites in rat brain. QA receptors are usually colocalized with NMDA receptors whereas KA sites have a more dissimilar distribution preferentially binding in the hippocampal CA3 stratum lucidum and granule cell layer of the cerebellum (Unnerstall and Wamsley 1983; Monaghan and Cotman 1982; Monaghan et al. 1984a; Rainbow et al. 1984). Voltage clamp studies (Mayer and Westbrook 1985; MacDermott et al. 1986) suggest that QA/KA receptors are associated with conventional conductances involving  $\text{Na}^+$  and  $\text{K}^+$ , with reversal potentials near zero (Mayer and Westbrook 1985). These conductances are voltage-independent and lead to fast depolarizations when activated by transmitters such as L-glutamate (MacDermott et al. 1986). QA and KA responses are also unaffected by changes in external  $\text{Ca}^{2+}$  (Ascher and Nowak 1986) or external  $\text{Mg}^{2+}$  (Ault et al. 1980). More recent studies have shown that the QA/KA antagonist CNQX (Honore et al. 1988; Blake et al. 1988) will block fast EPSPs at these synapses whereas the selective NMDA antagonists AP5 and AP7 have no effect (Evans et al. 1982).

### *3. Other Glutamate Receptors*

The fourth class of EAA receptor has been identified by utilizing the EAA antagonist L-AP4, the w-phosphonic derivative of L-glutamate (Körner and Cotman 1981; Foster and Fagg 1984). Experiments using spinal cord preparations (Davis and Watkins 1982) and rat cortical slices (Hearn et al. 1986) have demonstrated that this antagonist can block synaptic transmission in these systems. But recent studies (Cotman et al. 1986) utilizing guinea pig

hippocampal slices and rat olfactory cortex (Anson and Collins 1987), suggest that L-AP4 elicits its actions presynaptically in a chloride-dependent manner.

The fifth and most novel type of EAA receptor has been termed the APCD site by Monaghan et al. (1989) who have suggested that it may be coupled to phosphatidyl inositol metabolism. Initial evidence, provided by Sladeczek and his group (1985), demonstrated that EAAs stimulate the production of inositol phosphates in primary cultures of mouse striatal neurons. These results have been confirmed by other groups using rat brain cerebellar granule cells in culture (Nicoletti et al. 1986c), rat brain slices (Nicoletti et al. 1986a,b; Nicoletti et al. 1987a) and rat brain synaptosomes (Hollingsworth et al. 1985).

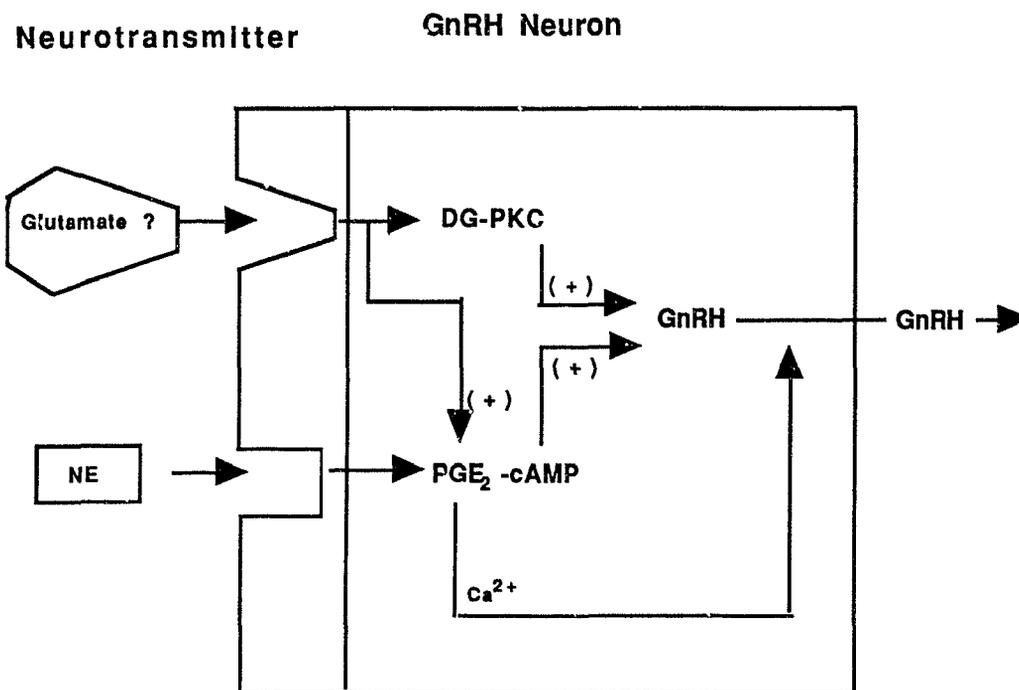
Experiments utilizing primary cultures of mouse striatal neurons (Sladeczek et al. 1985) indicate that quisqualate is the most potent EAA at the APCD receptor, although Monaghan et al. (1989) suggest that the glutamate analog APCD has a greater potency at this site. In an elegant set of experiments performed by Sugiyama et al. (1987) *Xenopus* oocytes were injected with rat brain mRNA which induced the expression of a particular glutamate receptor. These receptors were preferentially stimulated by QA, resulting in an increase in Cl<sup>-</sup> conductance. This response was mimicked by inositol-1,4,5-triphosphate (IP<sub>3</sub>) injection and blocked by EGTA, a calcium chelator, suggesting that the stimulation by QA resulted in IP<sub>3</sub> production and mobilization of intracellular Ca<sup>2+</sup> that activated Ca<sup>2+</sup> dependent Cl<sup>-</sup> channels. These researchers also demonstrated that NMDA had no effect on conductance nor did the NMDA antagonist APV block QA-

induced conductances. Interestingly, neither the QA antagonist GDEE nor Joro spider toxin (JSTX), specific blocker of ionotropic QA/KA receptors, blocked QA induced  $\text{Cl}^-$  conductances whereas the G protein inactivator, pertussis toxin (PT) inhibited the  $\text{Cl}^-$  conductance. Similar studies (Verdoorn and Dingledine 1988) using the same preparation, demonstrated that AMPA, the potent QA agonist, was ineffective in eliciting a response. These studies lend support to the work of Palmer et al. (1988) who demonstrated that CNQX, a selective QA antagonist, did not block QA-induced IP formation. This group also demonstrated that activation of the NMDA receptor appears to negatively regulate APCD-receptor-mediated IP production in neonatal rat hippocampal slices. Although the decrease in IP production could be a consequence of NMDA excitotoxicity, studies by Kushner et al. (1988) using rat brain mRNA-injected *Xenopus* oocytes, demonstrated that the NMDA-antagonist APV enhances the response of L-glutamate at the APCD receptor. This suggests that the NMDA receptor is negatively coupled to IP production.

The physiological role of the APCD receptor is speculative. Using rat hippocampal slices, Nicoletti et al. (1986a) demonstrated that ibotenate (QA agonist)-stimulated IP production progressively declines in immature rats between postnatal days 6 and 24. Similar results have been obtained using rat brain synaptosomes (Recasens et al. 1987), indicating that this receptor may play a significant role in synaptic growth and stabilization.

With respect to the theme of this thesis, the neurochemical activation of the completed neural circuitry resulting in pubertal

pulsatile GnRH release may also be mediated by the APCD receptor. Although Ojeda et al. (1986b) have suggested that the preovulatory surge of GnRH at puberty is activated by a norepinephrine (NE) - prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) - cAMP pathway, they have also demonstrated that a diacylglycerol (DG) - protein kinase C (PKC) pathway, whose neurotransmitter is unknown, also induces GnRH release (see Fig. 3). The simultaneous stimulation of both pathways within the GnRH neuron, resulting in an additive effect on GnRH release, suggests that activation of both pathways may be required for the proestrus LH surge to occur. It is of interest then, that studies by Wroblewski et al. (1987) and Nicoletti et al. (1987b), using primary cultures of neonatal cerebellar granule cells, have demonstrated that NMDA receptor activation results in increased phosphatidyl inositol hydrolysis, an effect that can be blocked by APV and regulated by Mg<sup>2+</sup>. These researchers have also demonstrated that L-glutamate is a potent stimulator of inositol phospholipid metabolism and, as for NMDA, the effect is antagonized by APV and regulated by Mg<sup>2+</sup>. Although these *in vitro* studies indirectly suggest a role for NMDA receptor activation in GnRH release more direct evidence for the involvement of this receptor will be discussed in following sections.



**Fig. 3.** Postulated intracellular pathways involved in the transduction of neurotransmitter signals leading to GnRH release during puberty in the female rat. In the afternoon of proestrus, increased activity of NE neurons activates the PGE<sub>2</sub>-cAMP system which acts, in combination with elevated Ca<sup>2+</sup> release to promote GnRH release. Another neurotransmitter system, possibly glutamate, may directly induce GnRH release or activate the PGE<sub>2</sub>-cAMP system by presumably activating the DG-PKC pathway. (NE) norepinephrine; (PKC) protein kinase C; (DG) diacylglycerol; (PGE<sub>2</sub>) prostaglandin E<sub>2</sub>; (cAMP) cyclic AMP; (Ca<sup>2+</sup>) calcium.

## D. Glutamate and Sexual Maturation

Numerous studies have suggested that glutamate is a regulatory component in the mature reproductive system. However, a more definitive role for this EAA in the process of sexual maturation is now being established.

### 1. *Influence of Neonatal Monosodium Glutamate*

Stimulation of hypothalamic glutamate receptors by monosodium glutamate (MSG) at birth results in abnormal puberty onset (Rodriguez-Sierra et al. 1980) and severe endocrinopathies (Olney 1969; Olney and Price 1980; Nemeroff 1984) in the adult. Early studies by Olney (1969) demonstrated that MSG, injected subcutaneously into neonatal rodents and monkeys (Olney et al. 1972) in high doses, produced lesions that were largely restricted to the ARC/N of the hypothalamus and the retina. Ablations in other circumventricular organs including the area postrema and the subfornical organ have also been seen (Oser et al. 1971). Histological examination of the mediobasal hypothalamus (MBH), using light and electron microscopy, indicates that 80-90% of the neuronal cell bodies are destroyed by MSG while glial cells and axonal processes passing through the nucleus are spared (Lemkey-Johnston and Reynolds 1974; Simson et al. 1977). Ultrastructural studies (Olney et al. 1972) have localized the action of glutamate to dendritic and somal portions of these neurons which contain the EAA receptors, through which the depolarizing effects of glutamate are mediated. The neurotoxicity of glutamate most likely occurs through sustained

activation of the NMDA-gated ion channel (Rothman and Olney 1986; 1987) resulting in a prolonged influx of  $\text{Ca}^{2+}$  ions causing neuronal degeneration and cell death (Choi 1987; Garthwaite 1989). The influx of  $\text{Cl}^-$  has also been implicated in NMDA-induced cell death (Rothman 1984), although, this appears to be a distinct property of tissue culture and probably results from unrestricted swelling (Garthwaite 1989).

The MBH plays a significant role in the regulation of anterior pituitary function, therefore, these well-defined lesions offer an opportunity to study chemically defined neuronal systems controlling the secretion of hypothalamic releasing or inhibiting factors into the portal blood system. Unfortunately the results of these studies are often conflicting, perhaps as a consequence of the different species that have been examined (Garratini 1979) or the various doses of MSG and injection schedules used (Seress 1982).

In an elegant and comprehensive study, Meister et al. (1989) used immunofluorescent histochemistry and autoradiography to examine the loss of a variety of neurotransmitters and neuropeptides in the arcuate nucleus of rats neonatally treated with MSG. These include: growth-hormone releasing factor, pro-opiomelanocortin-derived peptides (eg.  $\beta$ -endorphin and  $\alpha$ -MSH), enkephalin, dynorphin, galanin, neurotensin and neuropeptide Y. Glutamic acid decarboxylase (GAD) immunoreactivity also decreased, confirming earlier studies by Jennes et al. (1984). In agreement with an earlier study by Dawson (1986), Meister et al. (1989), found that MSG decreased dopamine (DA) levels in females, but had little effect in males. Dawson (1986), however, only observed DA decreases in

females after puberty; prepubertal values were normal. Using immunofluorescent histochemical studies, Marani et al. (1982) demonstrated that MSG treatment (2.5 mg/g on day 1 increased to 4.2 mg/g by day 11) of newborn female rats accelerates the migration of fluorescent dopaminergic perikarya from the median eminence to the arcuate nucleus between postnatal days 12 and 22. Subsequent to postnatal day 25, the arcuate becomes devoid of fluorescence suggesting that the DA perikarya have been destroyed and that DA cell loss may be a secondary effect of neonatal MSG treatment. Conversely, Walaas and Fonnum (1978) using immunofluorescence and uptake studies found that DA innervation in the arcuate was not significantly damaged by MSG treatment although both choline acetyl transferase and glutamate decarboxylase activities were severely compromised. This group used smaller doses of MSG (2 mg/g during the first week of life) than Marani et al. (1982) and failed to specify the sex of the treated animals from which they obtained their results. Using much larger doses of MSG, Meister et al. (1989), demonstrated that the cells that are sensitive to the neurotoxic effects of MSG are located in the ventrolateral and ventromedial areas of the arcuate nucleus whereas cell bodies in the dorsomedial area, such as those containing DA, are relatively insensitive. However, this group does concede that in densely innervated areas, immunohistochemical techniques may not be able to detect the loss of even a fairly large number of fibers.

Not unexpectedly, excitotoxic lesions in the arcuate nucleus result in numerous endocrinopathies when the animal eventually becomes sexually mature. Growth hormone (GH) synthesis and secretion are

greatly reduced (Nemeroff et al. 1978a; Terry et al. 1981; Corder et al. 1990) most likely resulting from the destruction of GH perikarya (Bloch et al. 1984). Corticosterone levels in MSG treated rats appear to be elevated (Olney and Price 1978) and are resistant to the suppressive effects of dexamethasone (Dolnikoff et al. 1988) suggesting a disrupted hypothalamic-pituitary regulation of adrenal function. Nemeroff et al. (1978a) found normal basal TRH and TSH levels in MSG- treated rats whereas tri-iodothyronine ( $T_3$ ) and free thyroxine ( $T_4$ ) were significantly reduced. These studies have been confirmed by others (Greeley et al. 1980) whereas Bakke et al. (1978) have demonstrated that neonatal MSG treatment results in a reduction of TSH in males and a significant reduction of serum  $T_4$  in females. This group rules out the possibility of primary hypothyroidism and suggests that the decrease in TSH may result from defective production secondary to the MSG-induced hypothalamic lesion.

There are numerous, but conflicting reports of the reproductive abnormalities associated with neonatal MSG treatment. Bakke et al. (1978) observed an increase in prolactin secretion (PRL) in females and an increase in basal levels of LH in males. Nemeroff et al. (1978b) originally reported that neonatal MSG resulted in an increase in PRL in males without effecting gonadotropin levels but later studies (Nemeroff et al. 1981) indicated that PRL was normal in both sexes but basal LH was decreased in the male and FSH was dramatically reduced in both males and females. Concentrations of the sex steroids, estradiol ( $E_2$ ) and testosterone, were also reduced. Neonatal MSG treatment results in acute degeneration of neurons in

the inner portion of the developing retina (Lucas and Newhouse 1957; Olney 1969; Olney et al. 1971; Rothman and Olney 1987). Therefore it is interesting that Nemeroff et al. (1981) observed that MSG-treated animals are blind even though they still exhibit a normal diurnal rhythm of pineal N-acetyl-transferase, which suggests that the reproductive abnormalities observed in these rats are not a result of impaired melatonin synthesis.

The post-castration rise of LH and FSH is delayed in both male and female rats neonatally-treated with MSG (Greeley et al. 1980; Clemens et al. 1978; Sridaran et al. 1981; Dyer et al. 1981) and is also more resistant to E<sub>2</sub>-induced attenuation (Dyer et al. 1981). These results correlate well with the studies of Rodriguez-Sierra et al. (1982) who demonstrated that E<sub>2</sub> receptors in the arcuate-ME area decrease after neonatal MSG treatment. They suggest that the loss of E<sub>2</sub> receptors induces the reproductive impairment observed in these animals. Jennes et al. (1984) have also demonstrated that the number of [<sup>3</sup>H]estradiol target cells in the arcuate nucleus is largely reduced after neonatal MSG treatment while those in the ventromedial nucleus are unaffected. They conclude that as a result of neonatal MSG treatment, certain populations of estradiol target cells in the arcuate are eliminated i.e. GABAergic and dopaminergic (Sar et al. 1983). It is possible that the loss of these cells may disturb the sensitive mechanisms of steroid feedback in both males and female rats, resulting in various reproductive anomalies.

Dawson et al. (1989) using high (4 mg/g on days 2, 4, 6 and 8) and low (4 mg/g days 2 and 4) doses of MSG observed no changes in vaginal opening (puberty) or estrous cyclicity when compared to

controls. Interestingly Rodriguez-Sierra et al. (1980), using the same high dose of MSG, observed a delay in VO. Bakke et al. (1978) using increasing doses of MSG from day 1 to day 5 observed no change in VO but did report irregular estrous cycles in treated animals.

Rose and Weick (1986) have demonstrated that MSG treatment results in hyperresponsiveness to the LH inhibiting action of the dopamine receptor agonists apomorphine and bromocriptine. They conclude that this represents denervation supersensitivity brought on by MSG-induced DA depletion (Nemeroff et al. 1977). This report (Rose and Weick 1987) also suggests that neonatal MSG treatment may lead to a reorganization of hypothalamic circuitry such that LH secretion normally regulated by the arcuate nucleus, is taken over by other hypothalamic structures. This interesting suggestion awaits confirmation.

Finally, Badger et al. (1982) treated neonatal male and female rats with MSG and examined the hypothalamic-pituitary responsiveness, between postnatal days 60 and 63, to exogenous GnRH, naloxone, and NMA (N-methyl-D,L-aspartate; 15 mg/kg). Their results indicated that basal levels of LH, FSH, and GnRH were unchanged in the MSG treated group but NAL, NMA and GnRH stimulated LH secretion was equal to or greater than in saline treated controls. These findings are unexpected for a number of reasons. First, adult male rats are usually relatively insensitive to NMDA stimulation (Cicero et al. 1988a; also see Sect I Results p. 95) although neonatal MSG treatment may somehow alter this sensitivity. Secondly, Olney and Price (1980) using a smaller dose of neonatal MSG and a larger dose of NMA (25 mg/kg) did not observe a change in LH secretion. They

reasoned that an intact arcuate nucleus (i.e. the presence of glutamate receptors) is required for NMA to induce LH release. Neonatal MSG treatment results in the destruction of arcuate nucleus cell bodies that possess glutamate receptors, therefore an NMA-induced LH response in these rats would not be predicted. It is evident that all of this work is in need of careful re-evaluation. Experiments relevant to these studies have been examined in subsequent sections of this thesis (see Sect. I Results p. 92).

## *2. Stimulatory Effects of Glutamate Agonists on LH Secretion in Non-lesioned Animals.*

If an excitatory mechanism is responsible for the neurotoxicity of glutamate and its analogs on the ARC/N (Olney and Price 1980), subtoxic doses of these compounds may result in accelerated neuronal firing comparable to the way iontophoretically applied glutamate stimulates neurons in other areas of the CNS. Olney et al. (1976) using male rats (postnatal day 55), were the first to demonstrate that subtoxic doses of glutamate (1000 mg/kg) injected subcutaneously (s.c.), rapidly (5-10 min) increased LH and testosterone levels. These results contrast with those of Nemeroff et al. (1978b) who, using the same experimental protocol as Olney et al. (1976), except with significantly heavier (older) rats, failed to demonstrate an increase in LH after glutamate injection. This group did observe a rapid transient increase in prolactin and a suppression of rhythmic growth hormone (GH) secretion supporting the results obtained by Terry et al. (1981). Carillo and Alcantara (1981) reported that in ovariectomized E<sub>2</sub>- and progesterone-primed adult

rats, acute glutamate treatment only increased LH when injected intravenously (i.v.) and not s.c.

To further explore the neuroexcitatory mechanism of glutamate-induced LH release, Price et al. (1978a) administered the glutamate analogs KA, NMDA and homocysteic acid (HCA) in low doses (1-100 mg/kg) to 25-day old male rats. Although s.c. injection of each compound resulted in a rapid elevation in LH, this group suggested that because NMDA is more metabolically inert than glutamate and less toxic and more specific than KA, it would be the most satisfactory molecule to use as a neuroendocrine probe.

The lowest effective stimulating dose (LED) of NMDA was 15 mg/kg and the lowest effective toxic dose (LETD) after hypothalamic examination was 50 mg/kg (s.c.) in 25-day-old male rats. Serum levels of LH peaked at 7.5 min after NMDA injection (25 mg/kg) and quickly dropped to control concentrations by 30 min. (Olney and Price 1980). Price et al. (1978b) reported that the LH releasing action of NMDA was not blocked by the dopamine (DA) antagonists pimozide or chlorpromazine. The cholinergic blocking agents atropine (muscarinic antagonist) and mecamylamine (nicotinic antagonist) when administered to 25 day old male rats also failed to suppress the NMDA-stimulated release of LH (Price et al. 1980).

Neither of the neuroinhibitory amino acids GABA or taurine by itself influenced serum LH concentrations (Price et al. 1978b; Masotto and Negro-Vilar 1987) but when injected with NMDA, both compounds blocked the rapid LH elevations observed 7.5 minutes after NMDA treatment. The GABA antagonist, bicuculline, when administered by itself does not alter LH secretion but did prevent

GABA from blocking the NMDA induced release of LH (Olney and Price 1980). This group has also shown that, although GABA and taurine can attenuate the NMDA-induced LH increase, the neurotoxic actions of this EAA analog and others could not be prevented. These results suggest that the inhibitory amino acids could be non-specific antagonists that exert their blocking actions (via hyperpolarization) other than at the NMDA site (Masotto and Negro-Vilar 1987), perhaps directly on the GnRH terminal itself (Olney and Price 1980).

Schanker and Cicero (1980) have demonstrated that the site of action of NMDA is probably hypothalamic. They incubated anterior pituitaries from 55 day old male rats with  $10^{-5}$ M NMDA or  $10^{-8}$ M GnRH. GnRH markedly stimulated LH release whereas NMDA was without affect. *In vivo* experiments (Cicero et al. 1988a) indicate that the NMDA-induced LH response was blocked by GPT-LHRH a GnRH antagonist, providing further evidence that this EAA analog mediates the increase in LH through the release of GnRH from the hypothalamus and not by acting directly at the pituitary.

More detailed studies are now possible with the availability of specific NMDA antagonists. Arslan et al. (1988) have shown that the NMDA-induced increase in LH in adult male rats can be blocked by the i.v. injection of the competitive NMDA antagonist APV. The possibility that the NMDA receptor is involved in the spontaneous release of hypothalamic GnRH is supported by the observation that APV (Arslan et al. 1988) or the non-competitive NMDA antagonist PCP (Boggon and Ondo 1989) when administered to castrated male rats, quickly reduced LH pulsatility.

Although the effects of NMDA/glutamate on LH secretion have been known for some time (Olney et al. 1976), it is only recently that studies have examined the role of excitatory amino acids as possible modulators of sexual maturation in rat (Urbanski and Ojeda 1987), sheep (Ebling et al. 1989) and monkey (Gay and Plant 1987). Earlier studies by Schainker and Cicero (1980) suggested an age related attenuation of the NMDA-induced LH response but more recent work by this group (Cicero et al. 1988a) have provided a more precise ontological picture. Their results indicate that NMDA (20 mg/kg BW) is modestly effective at elevating serum LH concentrations in male rats between postnatal days 10-15 but its peak effectiveness occurs between postnatal days 20-40 and then declines through maturation and becomes almost completely devoid of activity in 60-day-old rats. Dose response curves indicated that the relative dose required to produce half maximal stimulation of LH release (ED<sub>50</sub>) was 10-fold higher in 60 day-old male rats (37.8 mg/kg) than in prepubescent 30-day-old male rats (3.5 mg/kg).

Initial experiments on puberty in female rats were performed by Urbanski and Ojeda (1987) who infused NMDA (20 mg/kg i.v.) for 1 minute every half hour between 13:00 and 17:00 from day 26 to 29 in an attempt to produce plasma LH pulses of similar magnitude to those normally seen during the initiation of puberty. Their results indicate that NMDA, when pulsed discontinuously for 4 consecutive days leads to the development of peripubertal type LH secretion and precocious puberty (indicated by advancement of vaginal opening (VO) by 7 days). Similar results were obtained when GnRH (50 ng/kg) was delivered in this manner. In subsequent studies Urbanski

and Ojeda (1990) demonstrated that daily s.c. injections of MK-801, a potent, non-competitive inhibitor of NMDA actions (Monaghan et al. 1989) (day 26 until puberty) resulted in a delay of VO by approximately 4 days. When pubertal female rats are primed with E<sub>2</sub> (400 µg/ml in corn oil) a proestrus LH surge of preovulatory magnitude results. Using this model these researchers (Urbanski and Ojeda 1990) demonstrated that a single injection of MK-801 (0.2 mg/kg) at 12:00 or four i.v. injections (every half hour) of APV (30 mg) beginning at 11:30, suppressed the E<sub>2</sub>-induced surge, indicating that this antagonist-induced delay in puberty is mediated in part, by blocking the first preovulatory LH surge. Note however, that injection of MK-801 delayed, but did not prevent puberty. This suggests the possibility of another excitatory pathway or, alternatively, a compensatory increase in NMDA receptor activation which overcomes antagonist inhibition. Supporting this theory, Lopez et al. (1990) have demonstrated that intracerebroventricular administration of AP7 or DNQX attenuates the estradiol-induced LH surge in ovariectomized rats. They suggest that activation of NMDA receptors in the preoptic and non-NMDA receptors (KA) in the MBH are necessary for obtaining the estradiol-induced surge of LH secretion. Studies by Ondo et al. (1988), in which NMDA (50 pmol) was microinfused into different areas of the hypothalamus and subsequent changes in LH were measured, have lead to similar conclusions. These experiments demonstrated that NMDA stimulation of the medial preoptic area (MPO) and not the arcuate (ARC/N) results in increased serum LH concentrations. Conversely, *in vitro* experiments by Bourguignon et al. (1989b) using mediobasal

hypothalamic fragments (arcuate + median eminence) that contain GnRH terminals but not cell bodies, demonstrated that NMDA (50  $\mu$ M) rapidly induced GnRH secretion from this area of the hypothalamus. These experiments suggest an MBH site for NMDA-induced GnRH secretion and are discussed in more detail below.

The first experiments in the monkey examined the acute effects of EAA upon gonadotropin secretion (Wilson and Knobil 1982). They observed large rises in LH, FSH and prolactin after an i.v. injection of NMDA (15 mg/kg) in the adult female rhesus monkey similar to those observed in rat studies (Price et al. 1978a,b; Olney and Price 1980; Terry et al. 1981). Sexual maturation in higher primates is separated from birth and infancy (0-40 weeks) by a protracted period of prepubertal development during which time there is a hiatus in pituitary gonadotropin secretion and the gonads are held in a state of relative quiescence (Plant 1988). Testicular testosterone secretion is first observed at 2 1/2 years of age and spermatogenesis initiated between the third and fourth year of life (Plant 1988). Recent studies by Gay and Plant (1987) have attempted to interrupt this prepubertal inactivity by stimulating hypothalamic neurons with NMDA resulting in the release of GnRH and LH. Using castrated prepubertal male monkeys (13-18 months old) they demonstrated that a single i.v. injection of NMDA (15 mg/kg) increased LH 7-10 fold and FSH 4-5 fold, similar to increases produced by 0.3  $\mu$ g of GnRH. Pretreatment with APV blocked the NMDA-induced LH release but did not interfere with the LH releasing action of exogenous GnRH. Recent studies by Grosser et al. (1989) support this concept of NMDA-mediated GnRH secretion. Electrophysiological studies, using

ovariectomized adult rhesus monkeys with chronically implanted recording electrodes placed in the MBH, demonstrated that APV blocks the multiunit activity (volleys) associated with the initiation of gonadotropin hormone pulses (Wilson et al. 1984) and thus, inhibits the spontaneous activity of the hypothalamic pulse generator. Earlier primate studies (Yeoman and Terasawa 1984) measuring single unit activity (SUA) in the MBH used ketamine (20 mg), a non-competitive NMDA antagonist (Lodge et al. 1986) to sedate their animals during recording. Surprisingly, this group demonstrated that low doses of ketamine anesthesia did not block the progesterone-induced LH surge in ovariectomized E<sub>2</sub>-primed animals or the concomitant unit activity.

In an attempt to determine whether GnRH neurons in the hypothalamus of the prepubertal monkey may be prematurely provoked into producing a sustained train of intermittent GnRH release Gay and Plant (1988) used male castrated juvenile monkeys primed with GnRH (0.1  $\mu$ g/min for 3 min every hour) to enhance responsiveness of the gonadotrophs (Plant and Dubey 1984). Hourly injections (for 50 hours) of NMDA, 60 min after GnRH pituitary priming, resulted in LH discharges that were without decrement and of greater magnitude than those elicited by i.v. infusion of GnRH. This repetitive NMDA-induced excitation (14 mg/kg every 3 hours for 16-30 weeks) in intact juvenile males (Plant et al. 1989) resulted in premature activation of the hypothalamic-pituitary-Leydig cell axis and the initiation of spermatogenesis in animals that were only 19-22 months old i.e. a time at which untreated monkeys were still in the period of "hiatus". This precociousness could be blocked by

concomitant injection of the long acting GnRH antagonist GnRH-HOAc and was reversible following cessation of the pulses.

These findings have lead to the suggestion that GnRH neurons within the hypothalamus of immature primates are capable of being chemically driven in an adult manner. Thus, the prepubertal hiatus in intermittent GnRH/LH release cannot be accounted for by deficits in mechanisms within the GnRH neuron that govern the synthesis and release of the decapeptide. Nevertheless, this group has hypothesized that the absence of gonadotropin release during the prepubertal hiatus may be attributed to various properties of the GnRH neuron. They suggest that the secretory activity of GnRH neurons is uncoordinated (desynchronized) resulting in a constant but minute amount of releasing hormone being delivered to the pituitary. The action of i.v. injections of NMDA which produces repetitive pulsatile GnRH could result from the ability of NMDA to substitute for the developing neural circuitry which underlies the synchronous activation of GnRH neurons at other stages of development (infancy and puberty).

Thus, in rats (Urbanski and Ojeda 1987) and primates (Gay and Plant 1988), *in vivo* administration of EAA agonists and subsequent measure of LH release have provided an indirect method for studying the role of EAA in modulating the GnRH pulse generator during sexual maturation. Bourguignon et al. (1989 a) using hypothalamic explants and a static *in vitro* incubation system have been able to evaluate directly the putative change in GnRH secretion induced by EAA agonists and the different compounds that mediate agonist receptor binding. By injecting 50 day old male rats with 30

mg/kg of NMDA and then removing the hypothalamus these researchers demonstrated that the peak level of GnRH release induced by NMDA occurred 7.5 min after NMDA injection while the rise in serum LH occurred 10 min after injection. This differs from the results of Price et al. (1978a) who demonstrated that in 25 day old males LH reaches peak levels by 7.5 min and may be indicative of an age related desensitization of NMDA induced GnRH release (Cicero et al. 1988a). Bourguignon et al. (1989a) have also demonstrated that increasing concentrations of kainate or NMDA but not quisqualate resulted in a dose-related stimulation of GnRH secretion. The increase in GnRH release was abolished in the presence of D-600, a  $Ca^{2+}$  channel blocker. The stimulatory effect of kainate (50 mM) was not affected by increasing the  $Ca^{2+}$  concentration (from 1.8 mM to 4 mM) or increasing the  $Mg^{2+}$  concentration (from 1 mM to 2 mM). The addition of the NMDA antagonist APV or removing glycine from the culture medium, also had no effect on kainate-induced GnRH release (see Fig. 2 for diagram of receptor complex). Conversely, the stimulatory effect of NMDA (50mM) was blunted by the increasing  $Ca^{2+}$  and reversibly blocked by elevated  $Mg^{2+}$ . The presence of APV or the absence of glycine from the media also prevented NMDA-induced GnRH release. The concentration of EAA in this study should also be noted. Although NMDA and kainate at concentrations of 200  $\mu$ M and 25  $\mu$ M, respectively, cause acute reversible lesions in 15 day old chick embryo retina (Olney et al. 1986) the 50 mM concentration of both EAA used in this study did not result in neurotoxicity. Evidence for this was supported by experiments in which hypothalamic explants that were stimulated

every 15 minutes by NMDA produced GnRH pulses that had the same amplitude during eight successive stimulations. This result suggests that perhaps only a small fraction of the NMDA used is distributed to the receptors or, possibly, there is a low receptor sensitivity to the agonist (Cicero et al. 1988a) or that NMDA is non-toxic in rats of this age (see below).

Recent studies by Johnson and Ascher (1987) have demonstrated the facilitatory effect of nanomolar glycine (an inhibitory amino acid) concentrations on the electrophysiological response of cortical neurons to NMDA. This report is supported by Bourguignon et al. (1989a) who demonstrated that NMDA-induced GnRH release was maximal when the glycine concentration in the media was 10 nM and decreased when the glycine was increased (0.1-10  $\mu$ M). Interestingly, the content of brain kynurenic acid, a tryptophan metabolite and endogenous glycine antagonist, increases dramatically with age (Moroni et al. 1988) providing a possible explanation for the decrease in NMDA sensitivity in older animals.

In a subsequent study by Bourguignon et al. (1989b) the *in vitro* effects of different EAA antagonists on the pulsatile release of GnRH from the retrochiasmatic hypothalamus of 50-day-old male rats were examined. This group has previously shown that the release of GnRH from the retrochiasmatic hypothalamus *in vitro* is pulsatile under certain experimental conditions (Bourguignon and Franchimont 1984; Bourguignon et al. 1987). Using a similar experimental protocol, they demonstrated that when individual hypothalamic explants were incubated with various concentrations of MK-801 (0.01  $\mu$ M or 100  $\mu$ M),  $Mg^{2+}$  (2 mM), or glycine-free medium,

pulsatility was nearly abolished. When explants were incubated with the kainate antagonist DNQX, GnRH pulsatility was unaffected suggesting that kainate receptors may not be important for the physiological mechanism controlling GnRH secretion. This contradicts evidence presented by Donoso et al. (1990) who suggest that GnRH release does not result from NMDA receptor stimulation but, instead, is mediated through the KA/QA receptors, an effect that is blocked by DNQX but not the NMDA antagonist AP7. Some caution should be exercised when interpreting the results from this study. These researchers failed to include glycine in the incubation media and therefore the diminished NMDA induced release of GnRH would not be unexpected (Bourguignon et al. 1989 b). Note also, these researchers used adult male rats, a factor that may have contributed to the NMDA insensitivity and will be discussed later in this thesis (see Sect. I Results p. 95).

Although these studies emphasize the importance of the contents of the culture medium for studying pulsatile GnRH *in vitro*, more importantly, it suggests that the intrahypothalamic  $Mg^{2+}$  and glycine concentrations may play an important regulatory role in the mechanism of pulsatile GnRH release. The use of isolated tissue explants *in vitro* is also significant. The retrochiasmatic hypothalamus is defined by coronal cuts (3 mm in depth) made at the caudal border of the optic chiasm and the caudal end of the mammillary bodies with sagittal cuts along the lateral hypothalamic sulci. Thus, this area of the hypothalamus is deafferented from the preoptic area, the site of GnRH cell bodies (Barry et al. 1985; Wray and Hoffman 1986b; Silverman 1988), as well as from the rest of the

brain, and yet is capable of generating GnRH secretory pulses. This suggests that the secretory activity of the severed GnRH axons can be modulated through receptors sensitive to NMDA. This hypothesis is supported by electrophysiological studies of the isolated arcuate area (from ovariectomized-E<sub>2</sub> supplemented prepubertal rats) that has been maintained *in vitro* (Yeoman and Jenkins 1989). Electrical recordings from this tissue indicate an E<sub>2</sub>-dependent increase in neuronal activity during the afternoon (16:00 hr) that correlates with the time of day that LH rises in intact adults. These results suggest that interneurons located within the arcuate nucleus may be important for controlling GnRH pulsatility. Note also that the presence of NMDA receptors at these terminals is not compatible with the work of Ondo et al. (1988) which implicates the MPO and not the arcuate as the site of NMDA action (see earlier).

### **E. Current Work**

The experiments to be described in the Materials and Methods below were contemporary to those of Urbanski and Ojeda (1990), already described. Some of this work is published in abstract form or is In Press. Preliminary experiments examining the effect of glutamate agonists and antagonists on the induction of the immediate-early gene, c-fos, have also been presented in this thesis.

## Materials and Methods

### *Animals*

Litters of newborn (day 0) female Sprague Dawley rat pups with their mothers were obtained from Canadian Hybrid Farms, Halls Harbour, Nova Scotia, Canada. The animals were housed eight to ten per litter in plastic cages under controlled lighting (lights on from 0700-2100 h) and allowed free access to food and water. Litters were weaned at 21 days of age then housed 4-6 pups per cage. All rats were weighed at weekly intervals and were examined for vaginal opening (V.O.) beginning on day 25. At V.O. rats were weighed, killed and ovarian weights obtained along with numbers of eggs or corpora lutea. For some experiments male and female rats were obtained at age 21 days or 60 days and housed 4-6 rats per cage.

### *Drugs*

Monosodium glutamate (MSG) was generously provided by Dr. D. Nance (Dept. of Anatomy, Dalhousie University). Dextrorphan hydrochloride was a gift from Hoffman-La Roche (West Mall, Etobicoke, Ontario). N-methyl-D-aspartate (NMDA) and 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14624) were obtained commercially from Sigma Chemical Company, St. Louis MO. MK-801 and 2-amino-5-phosphonovalerate (APV) were purchased from Research Biochemicals Inc., Natick, MA. Pregnant mare serum was provided by the National Hormone and Pituitary Distribution Program (Dr. S. Raiti).

### *Collection and Processing of Blood*

Rats were quickly decapitated and trunk blood was collected in 12X75 mm glass culture tubes. Blood was stored for 2 hr at 4°C and then centrifuged at 2000 rpm for 30 min at 4°C. The resulting sera was transferred to 1.5 ml Eppendorf tubes and stored at -20°C until the LH was quantified by RIA.

### *Double Antibody Radioimmunoassay (RIA) of Serum LH*

The concentration of LH in each sample was determined by RIA using a kit supplied by the NIDDK and the National Hormone and Pituitary Distribution Program (University of Maryland Baltimore, Maryland U.S.A.).

### *Buffers*

The LH assay buffer (0.1 M sodium phosphate in 0.15 M NaCl) was comprised of the following: 1 L saline, 1.0 g gelatin, 0.189 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1.225 g  $\text{Na}_2\text{HPO}_4$  and 0.1 g thimerosal. The pH of the buffer solution was adjusted to 7.6 using 4.0 N NaOH. NRS (normal rabbit serum) was similar to the LH assay buffer except that it contained 5.0 ml of NRS and 18.3 g of disodium EDTA and no gelatin. The pH was adjusted to 7.6 using 4.0 N NaOH.

### *Assay Reagents*

The LH standard used was LH RP-3. The first antibody was anti-rat LH S-10. LH I-8 (100 µg/ampoule) was iodinated (see below) and used as the labeled hormone in the assay. The second or precipitating

antibody was goat anti-rabbit IgG serum and was obtained from Dr. Will Moger, Dept of Physiology and Biophysics, Dalhousie University.

#### *Radioiodination of LH*

Twenty microliters of LH I-8 was added to a vial of 1 mCi of Na[<sup>125</sup>I] with 50µl of 0.5 M sodium phosphate buffer (pH=7.6). To this, 25 µl of freshly prepared chloramine-T solution (0.7 mg/ml of 0.05 M phosphate buffer) was added to the reaction vial. After 90 seconds the reaction was terminated by the addition of 50 µl of sodium metabisulfite (2.4 mg/ml of 0.05 M phosphate buffer) directly to the reaction vial. After further dilution with 200 µl of LH assay buffer, the contents of the vial were transferred with a syringe to a disposable 10 ml pipette packed with Sephadex G-75 superfine (Pharmacia; 1 g Sephadex/60 ml LH buffer) that had been soaked in LH buffer overnight and replaced with fresh LH buffer the next day. The column was eluted with LH assay buffer and 0.5 ml fractions were collected in 12x75 glass culture tubes. Ten microliter aliquots of each 0.5 ml fraction were counted on a LKB Clinigamma 1272 gamma counter. The fraction showing the best binding (Fig. 4) as predetermined by binding tests was used as the radiolabel in the assay. A binding test was performed with total count (TC) tubes, non specific binding (NSB) tubes (no first anti-body) and zero tubes to determine the amount of specific binding.

#### *Assay Procedure*

A series of standards were prepared by serial dilution using the standard LH RP-3. The concentration of these standards ranged from

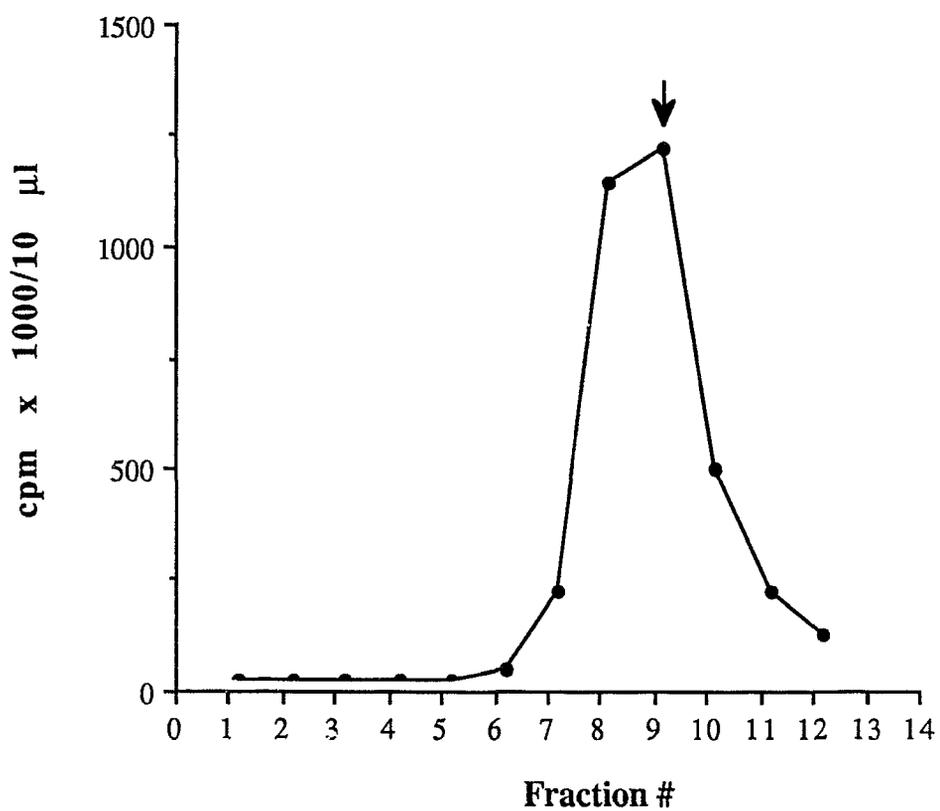


Fig. 4. Elution profile of  $^{125}\text{I}$ -labeled LH from an 18.0 cm x 0.9 cm column of Sephadex G-75 superfine. Fraction marked with an arrow was collected and used for the LH assay.

0 to 10 ng of LH RP-3 per 100  $\mu$ l. Samples consisted of 100  $\mu$ l of standard or 100-200  $\mu$ l of rat serum and were brought to a final volume of 500  $\mu$ l with LH assay buffer and assayed in duplicate. First antibody (LH S-10) diluted to a final concentration of 1:180,000 with NRS buffer and was added in a volume of 200  $\mu$ l to all tubes except those measuring TC and NSB. Tubes were vortexed and allowed to remain at 25°C for 72 hr. The radioactive label was then added in 100 $\mu$ l aliquots containing 25,000-35,000 cpm each. The tubes were vortexed again and placed at 25°C for 24 hr. After this incubation the second antibody (2.34 ml of Marigold serum), which was made up in a solution of LH assay buffer with 2.993 g of polyethylene glycol (PEG) per 32.66 ml of buffer, was added to the tubes as 200 $\mu$ l aliquots. The tubes were vortexed and allowed to sit at room temperature for two to three hours. After the incubation period 1.0 ml aliquots of LH assay buffer were added to the tubes. The tubes were centrifuged at 2000 rpm for 30 min at 4°C in an IEC DPR-6000 centrifuge after which the supernatant was decanted and the pellet remaining in the tube was then counted in the gamma counter. Non-specific binding was determined by incubating a set of tubes in the absence of sample and the first antibody. Duplicate samples from a rat serum pool (see below) were included in each assay.

#### *Calculation of LH in Samples*

LH concentrations were obtained by the log-logit method. Briefly, a linear regression is performed on the data obtained when the log<sub>10</sub>

of the concentration of the standards is plotted against logit  $B/B_0$ .

This factor is defined as:  $\text{logit } (B/B_0) = \ln[B/(B_0 - B)]$

where:

$B_0$  = the number of cpm in the absence of LH standard

$B$  = the number of cpm in the presence of increasing amounts of LH

The regression line is used to calculate LH concentrations from cpm. The assay sensitivity was 0.039 ng/100  $\mu$ l of LH and the intra-assay coefficient of variation was 6.0%. The inter-assay coefficient of variation was 11.5%. The serum pool was obtained from castrated adult male rats (2 weeks post-surgery) and was assayed at two dilutions (x1 and x5). All samples from each experiment were assayed together in a single assay. Values are expressed in terms of ng rat LH RP-3 (mean  $\pm$  S.E.M.)/ml (7-12 rats per group).

### *Ovariectomy*

Rats were ovariectomized on day 28 using ether or Equithesin anaesthesia and used experimentally 48 hr later. Ovaries were aseptically removed through a unilateral flank incision. The abdominal wound was closed with 1-2 catgut sutures and the initial incision was closed with 2 skin clips.

### *Laparotomy*

To inspect ovaries for the presence of eggs, a unilateral flank incision was made in rats anaesthetized with ether. The abdominal

wound was closed with 1-2 catgut sutures and the initial incision was closed with 2 skin clips.

## Experiments

### 1a. *Effect of Dextrorphan on Sexual Maturation*

Beginning at age 27 days, groups of immature female rats (n=10) were injected (s.c.) with the non-competitive glutamate antagonist, dextrorphan (18 mg/kg, in saline). Injections were made between 10:00 and 11:00 hr each day; control rats were injected with saline (0.1 ml). Treatment was continued until V.O. was observed. In our colony rats reach first ovulation coincident with V.O. in greater than 90% of the population.

### 1b. *Effect of APV on Sexual Maturation*

Beginning at age 27 days, groups of immature female rats (n=10) were injected s.c. with the competitive glutamate antagonist, APV (1 mg/kg in saline; 0.1 ml). Injections were made at 11:00 hr each day; control rats were injected with saline (0.1 ml). Treatment was continued until V.O. was observed.

### 1c. *Effect of MK-801 on Sexual Maturation*

Beginning at age 27 days, groups of immature female rats (n=10) were injected (s.c.) with the non-competitive glutamate antagonist, MK-801 (0.1 mg/kg, in saline). Injections were made at 11:00 hr each day; control rats were injected with saline (0.1 ml). Treatment was continued until V.O. was observed.

## 2. *Effect of Different Injection Times of MK-801 on Sexual Maturation*

To determine whether the time of injection was critical to the effect of MK-801 on sexual maturation, groups of immature female rats (n=10) were injected (s.c.; beginning on day 27) with MK-801 (0.1 mg/kg) at times throughout the day. Injections were made at 09:00 hr, 11:00 hr or 14:30 hr; control rats were injected with saline (0.1 ml). In another experiment MK-801 (0.1 mg/kg; sc) injections began on day 30 (11:00 hr) instead of day 27. All treatments were continued until V.O. was observed.

## 3. *Effect of MK-801 on Estrous Cyclicity*

Adult female rats which had demonstrated two consecutive estrous cycles were injected with MK-801 each day at 12: 0<sup>o</sup> hr beginning on the day of estrus. The dose was increased from 0.1 mg/kg to 0.5 mg/kg.

## 4. *Effect of Dextrorphan on PMS-induced Ovulation in Immature Rats*

The ability of dextrorphan to prevent first ovulation in rats stimulated with pregnant mare serum (PMS) was examined. Rats were received at 21 days of age. On day 25 at 11:00 hr a large group (n=20) of rats were injected with PMS (5 i.u.; s.c.; 0.1 ml). A subgroup (n=10) was treated with dextrorphan (18 mg/kg) at 10:30 hr and 13:30 hr on the day of proestrus. In this model, proestrus occurs on day 27. All rats were examined for the presence of ova/corpora lutea on the morning of day 28 (presumptive estrus).

### 5. *Effect of MK-801 on PMS-induced Ovulation in Immature Rats*

The ability of MK-801 to prevent first ovulation in rats stimulated with PMS was examined. Rats were received at 21 days of age. On day 25 a large group (n=21) of rats were injected with PMS (5 i.u.; s.c.; 0.1 ml). A subgroup (n=11) was treated with MK-801 (1 mg/kg) five minutes prior to PMS injection. Injections of MK-801 were repeated on days 26 and 27 (1 injection per day). All rats were killed and examined for ova/corpora lutea on the morning of day 28 (presumptive estrus). This experiment was repeated using a lower dose of MK-801 (0.1 mg/kg; s.c.). In another experiment a single injection of MK-801 (1 mg/kg; s.c.) was given at 14:00 hr on day 27 (proestrus). Animals were examined for ova/corpora lutea 24 (by laparotomy) and 48 hr after MK-801 injection.

### 6. *Effect of NMDA on Sexual Maturation*

Beginning at age 27 days, groups of immature female rats (n=10) were injected (s.c.) with the glutamate agonist, NMDA (15 mg/kg, in saline). Injections were made at 11:00 hr each day; control rats were injected with saline (0.1 ml). Treatment was continued until V.O. was observed.

### 7. *Effect of NMDA and MK-801 on Sexual Maturation*

In an attempt to reverse the influence of MK-801 on the timing of V.O., groups of immature female rats (n=10) were injected (s.c.; beginning on day 27) with NMDA (15 mg/kg, in saline) or NMDA followed 10 min later by injection of MK-801 (0.1 mg/kg). Injections

were made at 11:00 hr each day; control rats were injected with saline (0.1 ml). Treatment was continued until V.O. was observed.

#### 8. *Effect of Neonatal MSG on Sexual Maturation*

Four litters of 8-10 female pups were treated with MSG (4 mg/g; s.c.; 0.1 ml) or saline at 2, 4, 6 and 8 days after birth, 2 and 4 days after birth or 2 days after birth. Each litter was subdivided into two groups, of treated and control pups. Body weights were recorded at regular 4-5 day intervals until V.O. was observed. In subsequent experiments MSG treatment varied in both dose and day of injection. The dose of MSG was reduced to 1 mg/g or 2 mg/g, injected on postnatal day 2. The dose was further reduced to 0.25 mg/g injected on postnatal days 2 and 4 or postnatal day 2. In a final experiment, MSG (4 mg/g) was injected on postnatal days 10 and 12.

#### 9. *Effect of Neonatal MK-801 and MSG on Sexual Maturation*

In an attempt to prevent the toxic effects of MSG, rat pups were injected with MK-801 (1 mg/g; s.c.; days 2 and 4); MK-801 followed 10 min later by an injection of MSG (4 mg/g; days 2 and 4) or MSG alone. Body weights were recorded at regular 4-5 day intervals until V.O. was observed.

#### 10. *Effects of NMDA on LH Levels in the Immature Rat.*

The time course of the effects of NMDA and the effect of different doses of NMDA on serum LH levels in juvenile and adult *male rats* has been described in detail elsewhere (Schainker and Cicero 1980; Cicero et al. 1988a). In the majority of studies within this thesis

juvenile *female rats* have been used, therefore it was important to determine the time course and dose response of NMDA on LH secretion in these rats.

#### *Time Course*

Immature female rats (day 30; n=10/time point) were injected subcutaneously (s.c.) with NMDA (15 mg/kg) or saline at 11:00 hr and were decapitated at 0 min, 7.5 min, 15 min, and 30 min. post-injection. This dose of NMDA results in near-maximal LH secretion in male rats (Cicero et al. 1988a).

#### 11. *Dose Response*

The effect of different doses of NMDA on LH secretion was examined. Immature female rats (day 30; n=10/dose) were injected with NMDA (2, 5, 10, 15, and 20 mg/kg; sc) or saline and decapitated approximately 8 min later (based upon data from time-course experiment).

#### 12. *Ontogeny of the LH Response to NMDA*

In order to investigate the effect of age on NMDA-induced LH release in female rats, groups (n=10-15) were injected with NMDA (15 mg/kg; sc) beginning at day 4 and continuing through to day 32. Animals were killed 8 min post-injection and trunk blood was collected. In our colony the age of first ovulation (coincident with vaginal opening; V.O.) is approximately 34 days.

### 13. *Effect of MK-801 on LH Secretion in Ovariectomized Rats*

The ability of MK-801, a non-competitive blocker of the NMDA receptor, to inhibit the secretion of LH was examined in rats ovariectomized 48 hr previously. In these rats LH is sufficiently elevated that drug inhibition of LH secretion can readily be determined. Immature female rats (n=8/group) were ovariectomized on postnatal day 28 and injected with MK-801 (0.1 mg/kg) or saline 48 hr later. Animals were decapitated and blood collected 0.5, 1, 2, 3, and 4 hr following drug treatment.

### 14. *Effect of MK-801 on the LH Response to NMDA*

The ability of MK-801 to block the NMDA-induced LH surge was also examined. Immature female rats (day 30; n=10/group) were preinjected with MK-801 (0.1 mg/kg; s.c.) followed 1 hr later by an injection of NMDA (15 mg/kg; s.c.) or saline. Animals were decapitated and blood collected 8 min post-NMDA or saline injection.

### 15. *Effect of U-14,624 on the LH Response to NMDA*

The effect of catecholamine synthesis inhibition on naloxone-induced increases in LH secretion has been previously described. In the present work we examined the ability of U-14624, an inhibitor of dopamine  $\beta$ -hydroxylase (Johnson et al. 1970), to modify the NMDA-induced rise in LH release. Immature female rats (day 30; n=10/group) were preinjected with U-14624 (200 mg/kg; i.p.) or saline and injected 2 hr later with NMDA (15 mg/kg) or saline. Animals were decapitated and blood collected 8 min post-NMDA or saline injection.

#### 16. *Effect of Neonatal MSG on the LH Response to NMDA*

In this experiment, the neurotoxic effect of neonatal MSG treatment on the NMDA-induced LH surge in juvenile rats was examined. Twelve litters of 8-10 female pups were treated with different doses and injection schedules of MSG (4 mg/g on days 2, 4, 6 and 8; 4 mg/g on days 2 and 4; 1 mg/g on postnatal day 2) or saline. Each litter was injected with one of the MSG regimes or saline (three litters/treatment). On postnatal day 25 animals were injected with NMDA (15 mg/kg) or saline, decapitated 8 min later, and trunk blood collected. MSG effectiveness was determined by examination of the optic nerves in treated and control rats after sacrifice. The optic nerves of MSG treated rats are 50-70% smaller than those found in saline treated controls and therefore provide a valuable index of treatment effectiveness.

#### 17. *Effect of Ovariectomy on the LH Response to NMDA*

Immature female rats were ovariectomized on postnatal day 28. Half of the rats were injected with estradiol benzoate (EB; 10 $\mu$ g; in 0.1 ml sesame oil; injected at 14:30 hr) while the other half received saline. Forty-eight hours after surgery, EB and saline pretreated rats were injected with NMDA (15 mg/kg) or saline, decapitated 8 min post-injection and trunk blood was collected. Uteri were examined to check the influence of EB. Rats with bloated uteri were judged to be correctly primed with EB.

### 18. *Effect of Age on the LH Response to NMDA in Male Rats*

Juvenile (day 25; n=6/group) or adult (day 63; n=6/group) male rats were injected with NMDA (15 mg/kg) or saline, sacrificed 8 min post-injection and trunk blood was collected.

#### **Statistics**

Determination of significant differences between group means was accomplished using analysis of variance (ANOVA) followed by post hoc analyses using the Dunnett t-test with  $p < 0.05$  denoting a significant difference. These analyses were performed on an Apple MacIntosh II using Statview II (Abacus Concepts)

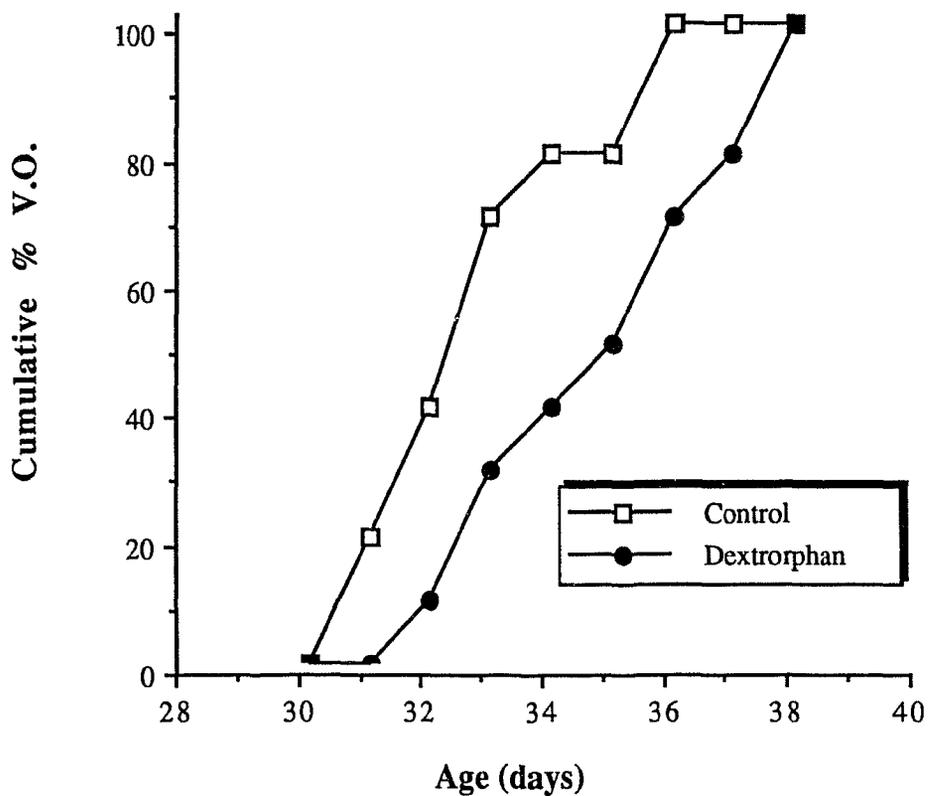
## Results

### 1. Effect of Glutamate Antagonists on Sexual Maturation

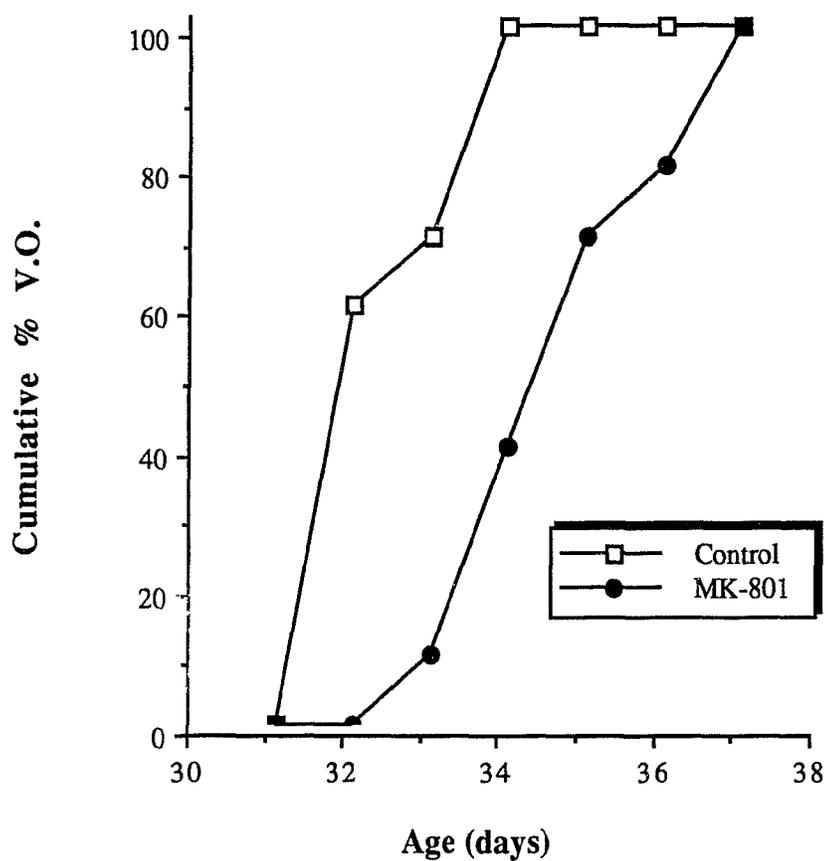
Figure 5 and 6 illustrate that V.O. is delayed, but not prevented, by dextrorphan or MK-801 treatment, respectively. The delay in V.O. was significant in both treatments and occurred at a larger body weight. (See Table 1). Conversely, the competitive antagonist, APV, had no effect on the timing of V.O. (Fig. 7) or body weight at V.O. (Table 1). In three separate experiments with MK-801 the number of rats that ovulated at V.O. were Con-28/30 and MK-801- 17/30. The ovulation rates for dextrorphan and APV treatment were 10/10 and 8/10, respectively. All remaining rats were in proestrus (bloated, fluid-filled uteri).

### 2. Effect of Different Injection Times of MK-801 on Sexual Maturation

The effect of different MK-801 injection times on the occurrence of V.O. is shown in Fig. 8. Daily injections at 09:00 hr (beginning on day 27) had no effect on the timing of puberty whereas injections at 11:00 hr and 14:30 hr significantly delayed V.O. Body weights at V.O. of animals injected with MK-801 at 9:00 hr were not different from controls whereas rats injected at 11:00 hr and 14:30 hr were significantly heavier (Table 2). Differences in mean ovarian weights and the number of C.L. at V.O. were not different from control and treated groups, although fewer MK-801-treated animals had ovulated at the time of V.O. (Table 2). When MK-801 injections were given from postnatal day 30 until V.O. (Fig. 9) sexual maturation was not effected.



**Fig. 5.** Effect of single, daily injections (day 27 until V.O.) of saline (n=10) or dextrophan (18 mg/kg; n=10) on the timing of V.O., plotted as the cumulative % of rats showing V.O.

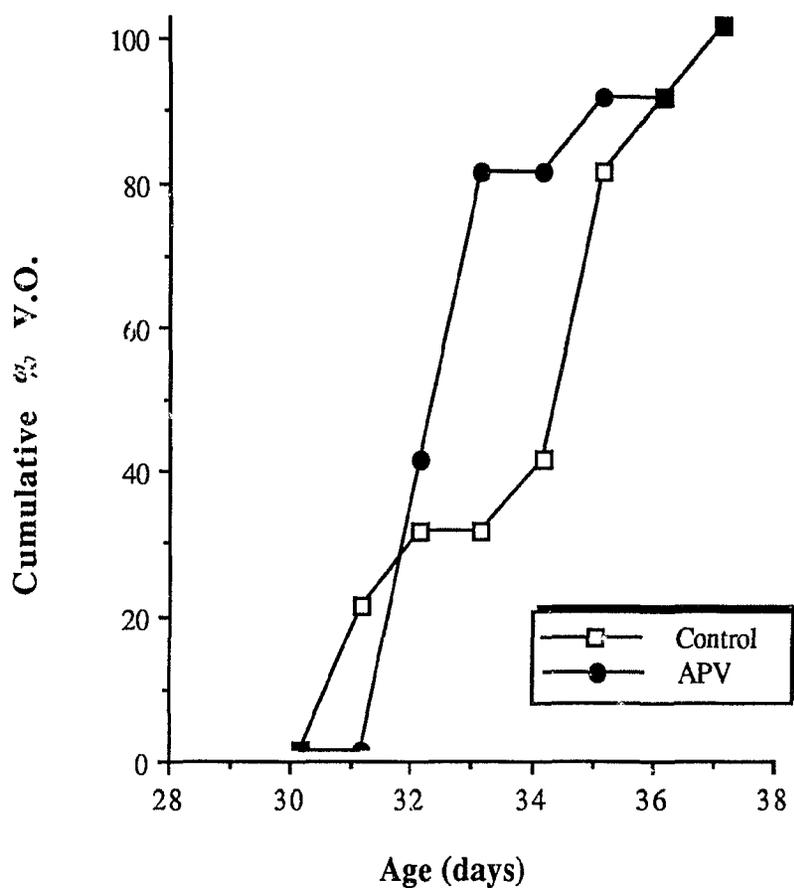


**Fig. 6.** Effect of single, daily injections (day 27 until V.O.) of saline (n=30) or MK-801 (0.1 mg/kg; n=30) on the timing of V.O. plotted as the cumulative % of rats showing V.O.

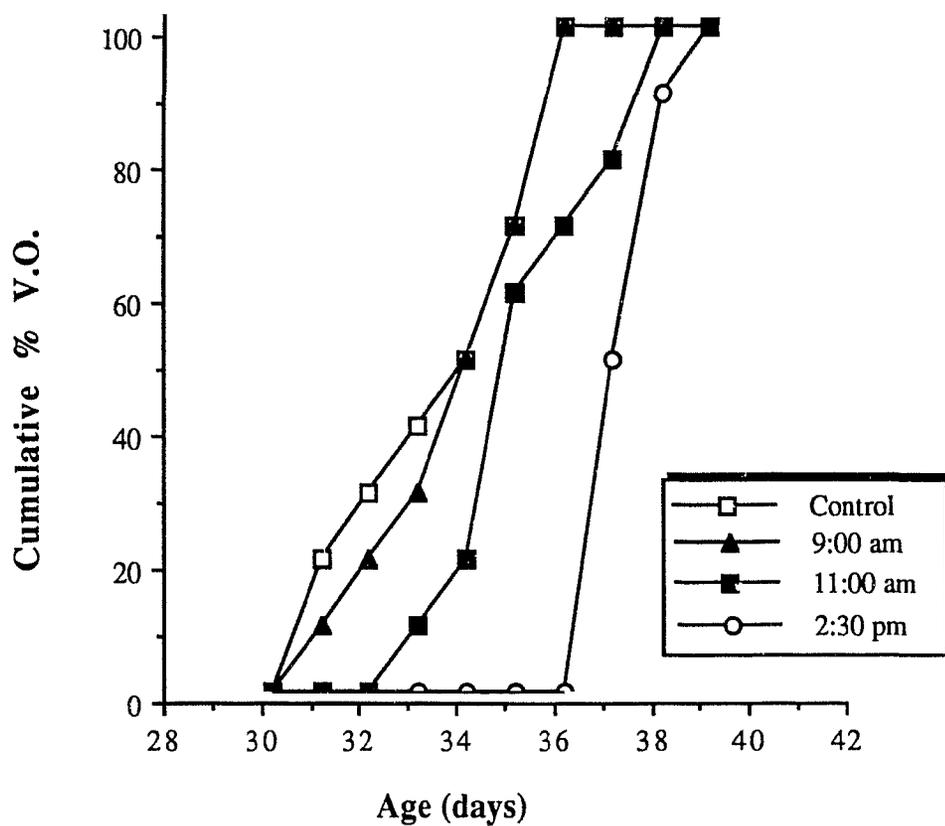
**Table 1.** Effect of different glutamate antagonists on age, body weight and the presence of ova at vaginal opening. Values are mean $\pm$ s.e.m.

Treatment	n	Age at V.O. (days)	Body Wt (g)	# having ova at V.O
Control	10	33.1 $\pm$ 0.6	113.8 $\pm$ 3.4	8/10
Dextrophan (18 mg/kg)	10	35.2 $\pm$ 0.7*	123.2 $\pm$ 3.6*	10/10
Control	30	32.7 $\pm$ 0.3	113.1 $\pm$ 2.6	28/30
MK-801 (0.1 mg/kg)	30	35.0 $\pm$ 0.4**	123.0 $\pm$ 2.0**	17/30
Control	10	33.2 $\pm$ 0.5	110.4 $\pm$ 3.4	8/10
APV (1.0 mg/kg)	10	34.1 $\pm$ 0.6	115.3 $\pm$ 5.0	8/10

\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; when compared to respective saline-injected controls; Student's t-test.



**Fig. 7.** Effect of single daily APV injections (1.0 mg/kg; from day 27 until V.O.) on the timing of V.O. (n=10). Control rats were injected with saline (n=10). Data plotted as the cumulative % of rats showing V.O.

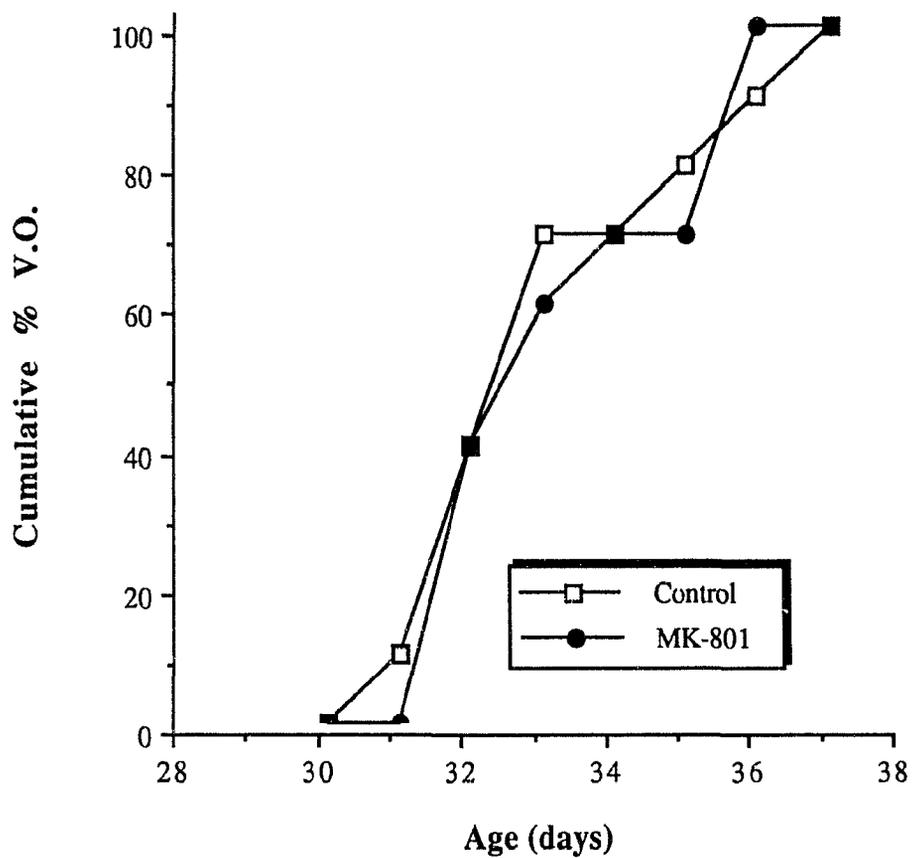


**Fig. 8.** Effect of single daily injections (from day 27 until V.O.) of MK-801 (0.1 mg/kg) given at 09:00 hr (n=10), 11:00 hr (n=10) or 14:30 hr (n=10) on the timing of V.O. Control rats (n=10) were injected with saline. Data plotted as cumulative % of rats showing V.O.

**Table 2.** Effects of MK-801 (0.1 mg/kg) injected at different times of the day on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean±s.e.m.

	Control	MK-801 (09:00 hr)	MK-801 (11:00 hr)	MK-801 (14:30 hr)
n (rats/group)	10	10	10	10
Age at V.O. (days)	33.9±0.6	34.2±0.5	35.8±0.4**	37.6±0.2***
Body Wt. (g)	103.2±3.2	103.0±2.6	114.2±1.2**	118.7±0.9***
Paired Ovarian Wt. (mg/100 g B.W.)	32.5±1.6	32.9±1.4	29.8±1.3	29.1±1.0
Total CL	10.8±0.2	11.1±0.5	10.6±0.3	11.0±0.5
# having ova at V.O.	9/10	10/10	8/10	7/10

\*\* p<0.01; \*\*\* p<0.005 when compared to saline-injected controls; ANOVA and post hoc Dunnett t-test



**Fig. 9.** Effect of single, daily injections (day 30 until V.O.) of MK-801 (0.1 mg/kg; n=10) or saline (n=10) on the timing of V.O. Data plotted as the cumulative % of rats showing V.O.

### 3. Effect of MK-801 on Estrous Cyclicity

A similar experiment was performed on adult, cycling rats. MK-801 (0.1 mg/kg) was injected once per day (11:00-12:00 hr), beginning on the day of estrus for 12 days. The rats had previously shown two complete estrous cycles. At this dose, no unusual behavioral effects were observed but estrous cyclicity was affected. In all animals the diestrous phase was extended by one day, lengthening the estrous cycle to five days. At a dose of 0.5 mg/kg for 16 days, MK-801 had profound behavioral and physiological effects. Animals appeared disoriented and uncoordinated for over 6 hr after injection. Body weight decreased, daily grooming ceased and all animals developed dark circles around their eyes. Estrous cyclicity was irregular in all animals with both diestrus and estrus being prolonged by 1-2 days. This lengthened the entire cycle to 7-8 days.

### 4. Effect of Dextrorphan on PMS-induced Ovulation in Immature Rats

An attempt was made to block PMS-induced first ovulation via injections of MK-801 or dextrorphan (Table 3). Dextrorphan (18 mg/kg), injected at 10:30 hr and 13:30 hr on the day of proestrus, had no effect on ovulation as determined by inspection of the ovaries next day. In the control group 9 of 10 rats ovulated whereas all of the treated rats ovulated normally. The number of ova and corpora lutea per rat were not different between the two groups.

**Table 3.** Effect of MK-801 or dextrorphan on PMS-induced ovulation. Values are mean $\pm$ s.e.m.

<b>Treatment</b>	<b>n</b>	<b>Body Wt (g)</b>	<b>Corpora lutea (C.L.)</b>	<b>% in Proestrus on Day 28</b>
<b>Control</b>	10	--	10.9 $\pm$ 0.4	0
<b>Dextrorphan (18 mg/kg) Inj. 10:30 hr and 13:30 hr on presumptive proestrus (day27)</b>	10	--	10.5 $\pm$ 0.2	0
<b>Control</b>	10	82.2 $\pm$ 1.1	9.8 $\pm$ 0.3	0
<b>MK-801 (1 mg/kg) Inj. 13:00 hr; days 25, 26 and 27</b>	11	69.5 $\pm$ 1.1***	0	100
<b>Control</b>	8	83.1 $\pm$ 1.5	9.6 $\pm$ 0.3	0
<b>MK-801 (0.1 mg/kg) Inj. 13:00 hr; days 25, 26 and 27</b>	7	89.1 $\pm$ 1.9	8.8 $\pm$ 0.5	0
<b>MK-801 (1 mg/kg) Inj. 14:00 hr; day 27</b>	8			
Inspected on Day 28:		--	0	100†
Day 29:		81.3 $\pm$ 1.6	8.9 $\pm$ 0.5	0

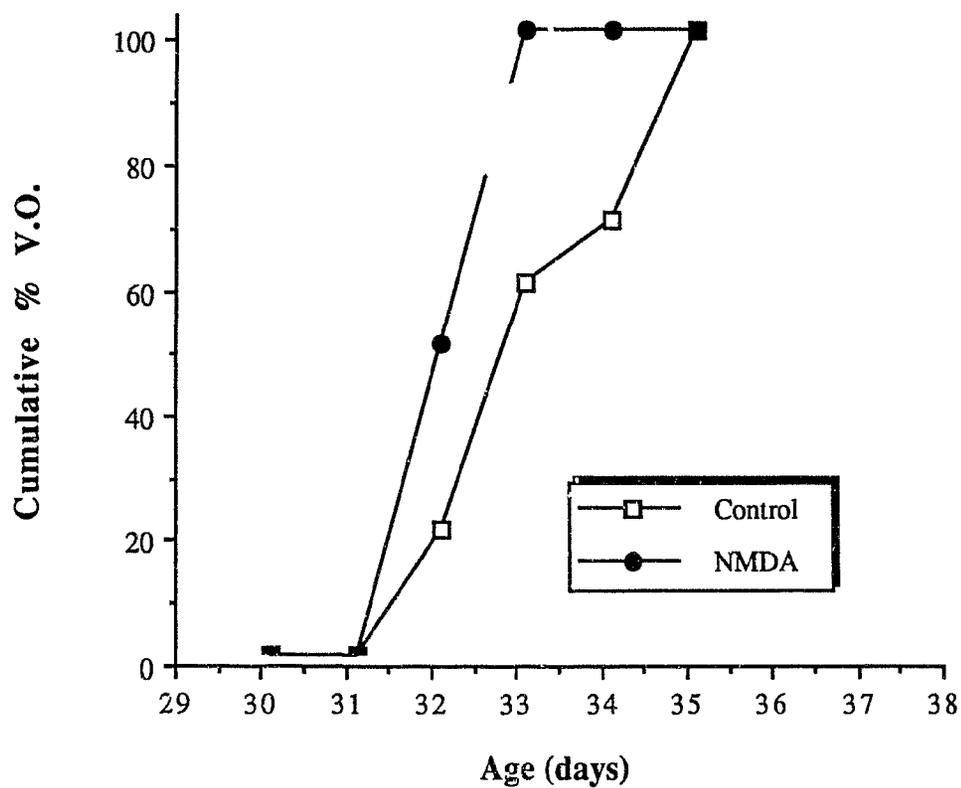
In PMS-induced ovulation, day 27 is considered presumptive proestrus and day 28 presumptive estrus. \*\*\*p<0.005; when compared to respective saline injected controls; Student's t-test †. determined by laparotomy.

### 5. Effect of MK-801 on PMS-induced Ovulation in Immature Rats

The above experiment was repeated with MK-801 (1 mg/kg) but this time injections were made (1 per day) on three consecutive days (i.e. at ages 25, 26, 27 days; 13:00 hr). Inspection of the reproductive tracts on the day of ovulation (day 28) revealed that all of the control rats ovulated normally whereas MK-801-treated rats were all arrested in proestrus. In subsequent experiments a single injection of MK-801 (1 mg/kg; s.c.) given at 14:00 hr on day 27, successfully prevented ovulation. All treated rats were in proestrus. However, when rats were treated with MK-801 on proestrus were then killed 48 hr later, freshly released ova were observed in the oviducts. Body weights and number of corpora lutea were not different from controls (estrus) values (See Table 3). Finally, a lower dose of MK-801 (0.1 mg/kg; s.c.; 1:00 pm; days 25-28) had no effect on PMS-induced ovulation. At the highest dose of MK-801 (1 mg/kg) adverse effects were observed, e.g. an ability to remain upright even though attempts were made to walk around. A reduction in body weight was also noted at 28 days (Table 3).

### 6. Effect of NMDA on Sexual Maturation

Figure 10 demonstrates the unequivocal acceleration of sexual maturation induced by single daily injections of the glutamate agonist, NMDA (15 mg/kg). Rats treated with NMDA weighed significantly less than controls at V O., although ovarian weights and number of C.L. did not differ between the two groups (Table 4). Note also that when the day of vaginal opening is plotted as % vaginal



**Fig. 10.** Effect of single, daily NMDA injections (n=10) (15 mg/kg; from day 27 until V.O.) or saline on the timing of V.O., plotted as the cumulative % of rats showing V.O.

**Table 4.** Effects of daily injections of NMDA (11:00 hr, beginning on day 27) on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

	Control	NMDA (15 mg/kg)
n (rats/group)	10	10
Age at V.O. (days)	33.8 $\pm$ 0.3	32.5 $\pm$ 0.1**
Body Wt. (g)	109.5 $\pm$ 1.1	101.5 $\pm$ 1.0***
Paired Ovarian Wt. (mg/100 g B.W)	31.9 $\pm$ 2.1	33.1 $\pm$ 1.7
Total CL	10.1 $\pm$ 0.2	10.5 $\pm$ 0.2

\*\*p<0.01; \*\*\* p<0.005 when compared to saline injected controls; Student's t-test.

opening per day (Fig. 11) there appears to be an NMDA-induced synchronization of vaginal opening. (see discussion).

#### 7. Effect of NMDA and MK-801 on Sexual Maturation

Figure 12 illustrates that the delaying effect of MK-801 (Fig. 6) could be reversed if NMDA was injected 10 minutes beforehand. MK-801 injection alone significantly delayed the age of sexual maturation whereas the combined NMDA-MK-801 treatment had no effect when compared to controls (Table 5). The mean body weight at V.O. of MK-801-injected animals was significantly heavier than controls, whereas body weights of the NMDA-MK-801 injection group were not different from controls. No differences in ovarian weights or number of C.L.s were observed among the different groups.

#### 8. Effect of Neonatal MSG on Sexual Maturation

Neonatal injections of MSG were based upon extensive published data (Nemeroff 1984; Olney and Price 1983). In an attempt to reduce the mortality rate MSG (4 mg/g; s.c.) was injected on days 2, 4, 6, and 8 of life. The majority of pups survived this treatment, which resulted in precocious sexual maturation (Fig. 13). Mean age at V.O., body weights as well as pituitary and ovarian weights were significantly lower in the treated group (Table 6). When expressed as means per 100g body weight, pituitary and ovarian weights did not differ from controls (see Table 6). This experiment was repeated with a lower total dose of MSG (4 mg/g; days 2 and 4 only; medium dose). This dosage is reported to produce extensive damage to the arcuate nucleus without damage to the preoptic area (Dawson 1986).

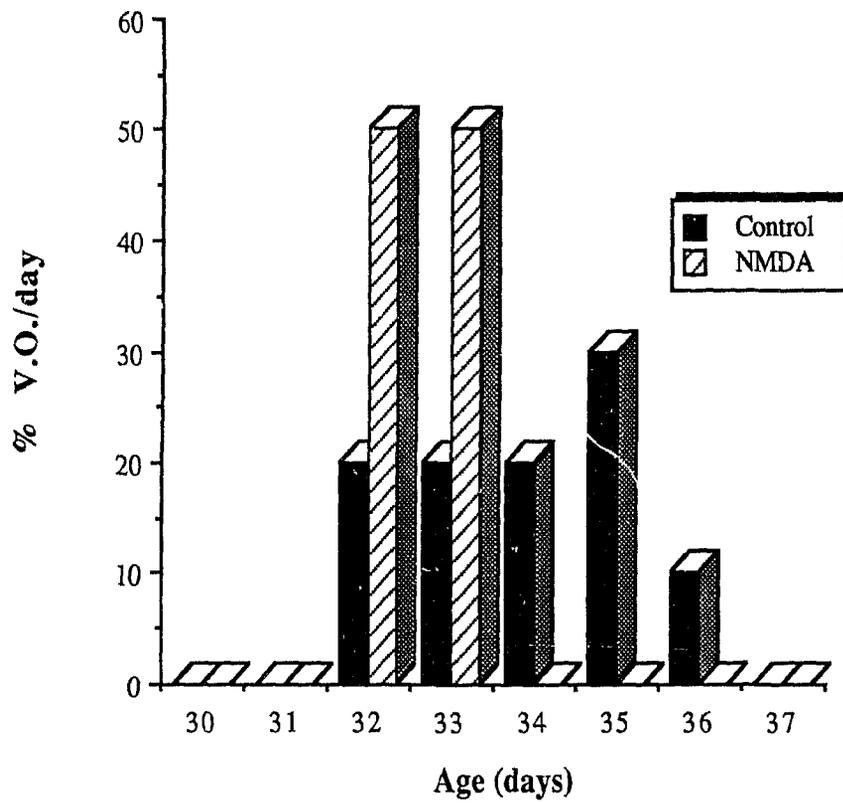
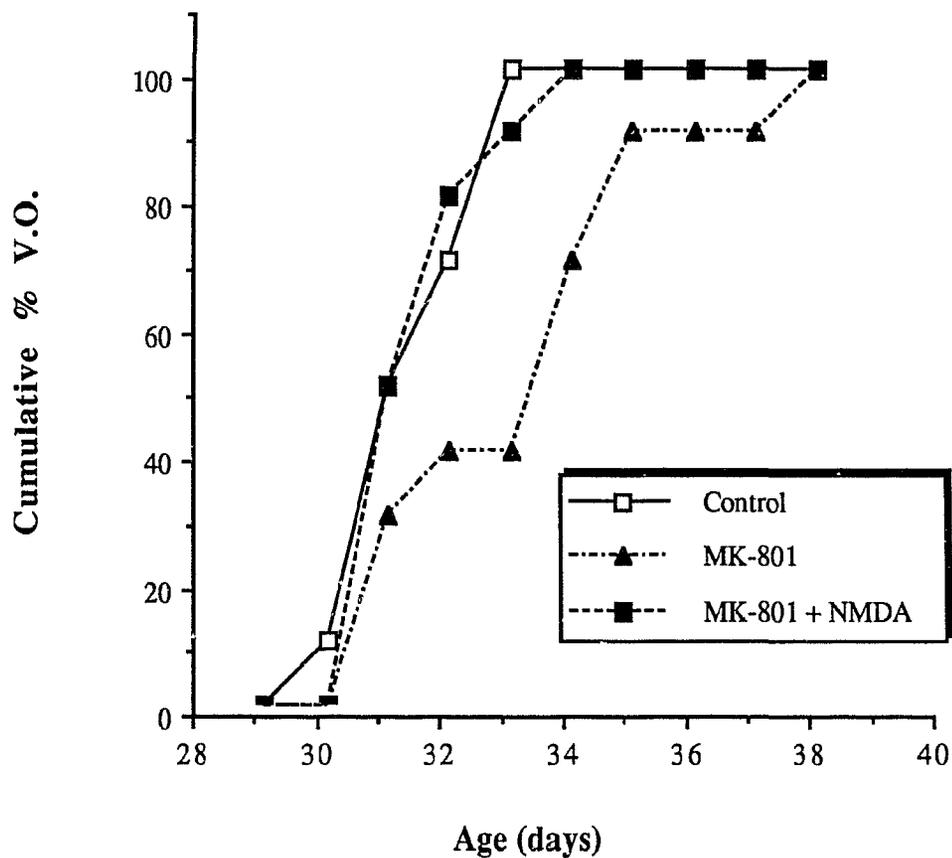


Fig. 11. Effect of single, daily NMDA injections (n=10) (15 mg/kg; from day 27 until V.O.) or saline (n=10) on the timing of V.O., plotted as the percentage (%) of rats showing V.O. at each age.

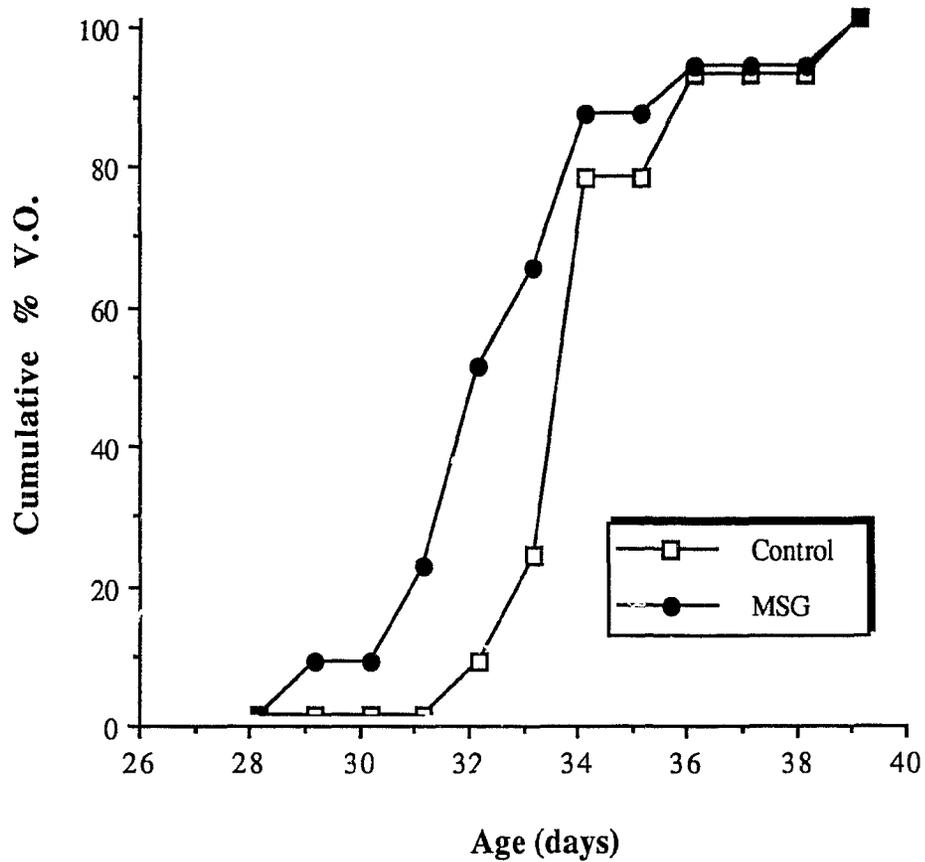


**Fig. 12.** Effect of pre-injection of NMDA (15 mg/kg) with MK-801 (0.1 mg/kg; n=10) beginning on day 27, on the MK-801-induced delay in V.O. (n=10) Control rats were injected with saline (n=10) Data plotted as the cumulative % of rats showing V.O. at each age.

**Table 5.** Effects of pre-injection with NMDA followed by an MK-801 injection on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

	Control	MK-801 (0.1 mg/kg)	MK-801 + NMDA (15 mg/kg)
n (rats/group)	10	10	10
Age at V.O. (days)	31.6 $\pm$ 0.3	33.5 $\pm$ 0.6**	31.8 $\pm$ 0.3
Body Wt. (g)	97.3 $\pm$ 2.1	106.6 $\pm$ 4.5*	101.0 $\pm$ 1.4
Paired Ovarian Wt. (mg/100 g B.W)	31.1 $\pm$ 1.6	29.0 $\pm$ 1.9	27.6 $\pm$ 0.8
Total CL	10.0 $\pm$ 0.5	11.4 $\pm$ 0.7	10.9 $\pm$ 0.4

\*  $p < 0.05$ ; \*\*  $p < 0.025$  when compared to control; ANOVA and post hoc Dunnett t-test



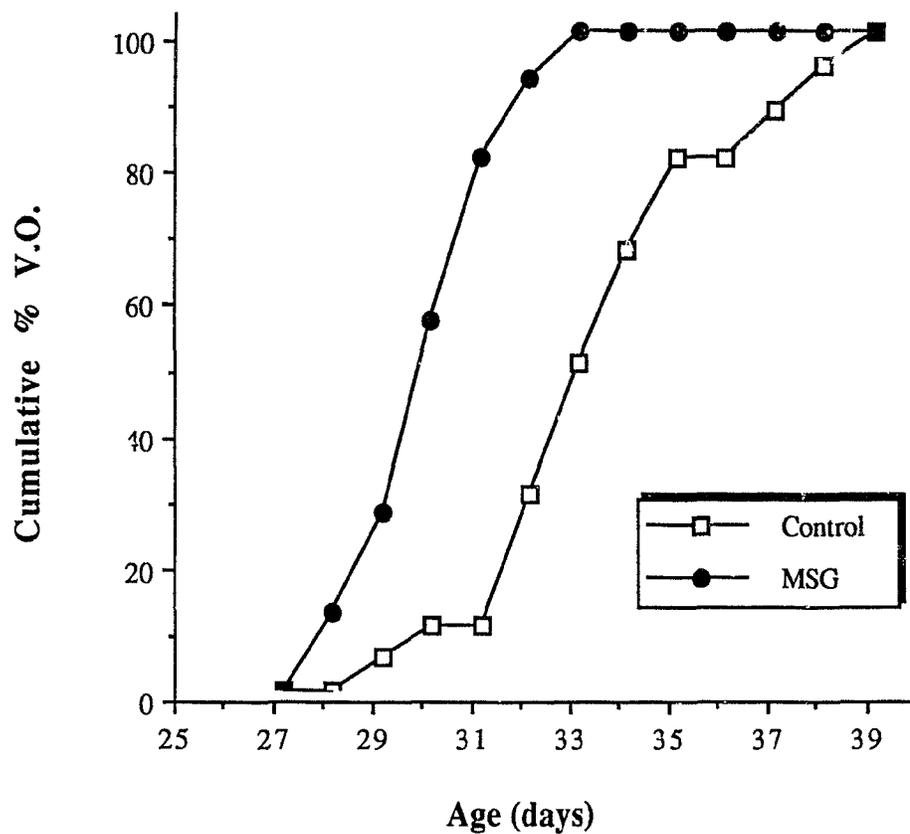
**Fig. 13.** Effect of MSG (4 mg/g body wt.), injected on postnatal days 2, 4, 6 and 8, on the timing of V.O. (n=13). Control rats (n=15) were injected with saline. Data plotted as the cumulative % of rats showing V.O.

**Table 6.** Effects of high (4 injections) dose, medium (2 injections) dose and low (1 injection) dose neonatal MSG on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

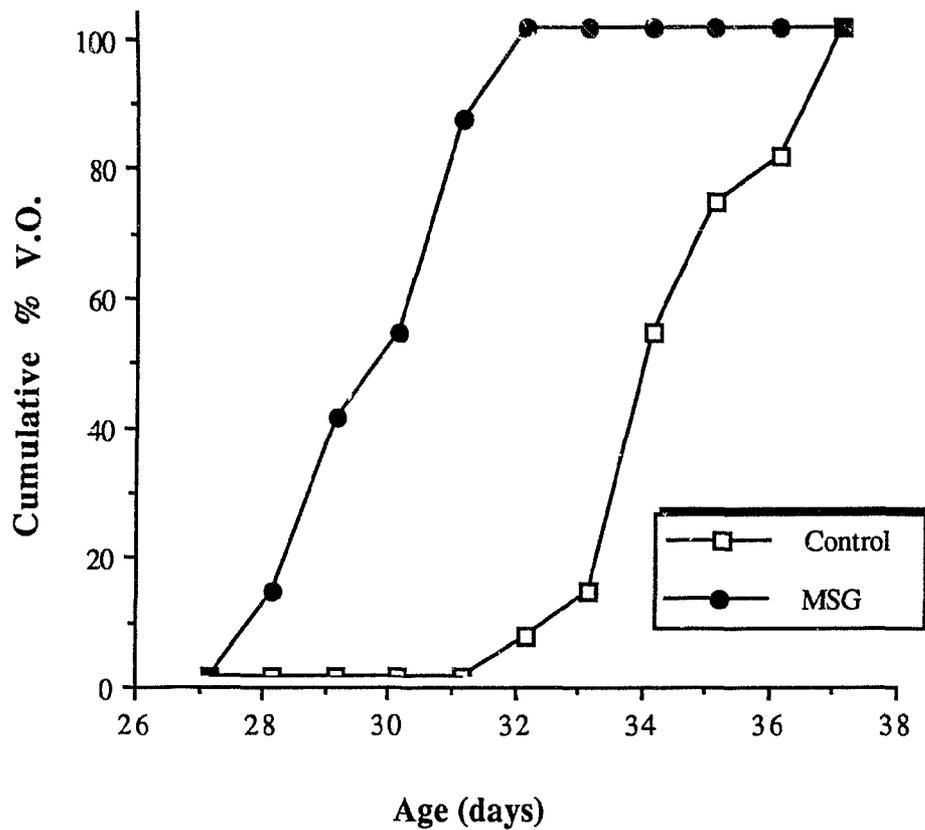
	Control	MSG (4mg/g; day 2)	Control	MSG (4 mg/g; days 2 and4)	Control	MSG (4 mg/g; days 2,4,6, and 8)
	Low Dose	Low Dose	Medium Dose	Medium Dose	High Dose	High Dose
n (rats/group)	15	15	23	25	15	13
Age at V.O. (days)	34.7 $\pm$ 0.4	30.0 $\pm$ 0.3***	34.5 $\pm$ 0.5	30.5 $\pm$ 0.6**	33.3 $\pm$ 0.5	31.8 $\pm$ 0.6*
Body Wt. (g)	130.3 $\pm$ 2. 7	97.9 $\pm$ 2.1***	112.6 $\pm$ 2.3	83.1 $\pm$ 3.2**	121.0 $\pm$ 2.6	100.1 $\pm$ 4.0**
Paired Ovarian Wt. (mg/100 g B.W)	28.9 $\pm$ 1.6	28.2 $\pm$ 1.0	36.9 $\pm$ 2.6	36.1 $\pm$ 1.9	29.1 $\pm$ 2.0	30.0 $\pm$ 1.3
Pit. Wt. (mg/100 g b.w.))	--	--	6.6 $\pm$ 0.2	5.9 $\pm$ 0.2	5.3 $\pm$ 0.3	4.2 $\pm$ 0.7
Total CL	13 $\pm$ 0.4	11.6 $\pm$ 0.5*	11.5 $\pm$ 0.4	11.4 $\pm$ 0.7	11.4 $\pm$ 0.9	8.8 $\pm$ 0.2

\* p<0.05; \*\* p<0.005; \*\*\*p<0.001 when compared to respective saline-injected controls; Student's t-test

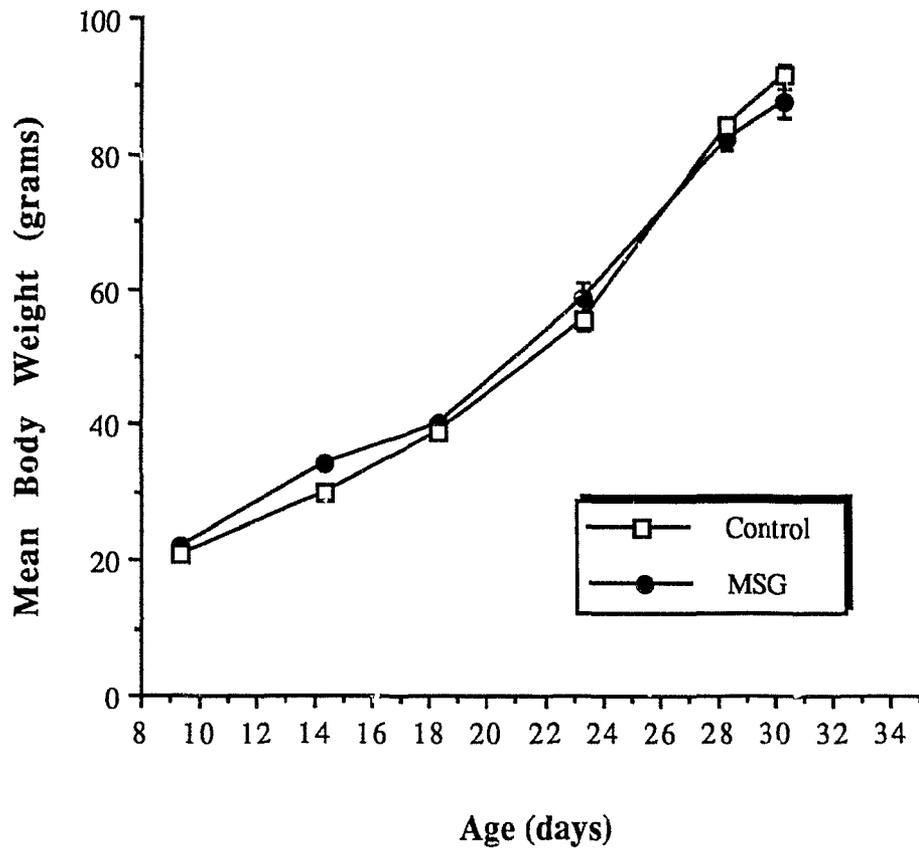
Figure 14 illustrates the results of this experiment. Treated rats reached first ovulation earlier and at a lower body weight. Again ovarian and pituitary weights were normal when expressed as means per 100g body weight (see Table 6). When the dose of MSG was reduced further (4 mg/g on day 2 only; low dose), treated rats ovulated earlier and at a lower body weight (Fig. 15). When expressed as mean per 100g body weight, ovarian weights were not significantly different. All rats, with the exception of two proestrous rats in the medium and high dose group and three from the low dose group, ovulated with a normal complement of ova and corpora lutea although estrous cyclicity was irregular. Body weight gain was identical in all of the MSG treated groups and control groups (Fig. 16; only medium dose presented). Precocious eye opening was also noted in treated rats. For example, at day 13, 20/23 MSG rats had eyes open with 8/25 rats in the control group. When the dose of MSG was reduced to 1 mg/g or 2 mg/g and injected on postnatal day 2, precocious V.O. was once again observed (Fig. 17). The mean age at V.O. using either dose of MSG was significantly earlier when compared to controls. The mean body weights of the two treated groups were also significantly reduced (Table 7). Interestingly, ovarian weights and number of C.L.s were not different from controls. In contrast, neonatal MSG treatment using doses of 0.25 mg/g injected on day 2 or days 2 and 4, had no effect on the timing of sexual maturation (Fig. 18) or mean body weights (Table 8). MSG treatment (4 mg/g) on postnatal days 10 and 12, did not induce precocious sexual maturation (Fig. 19) nor were body weights,



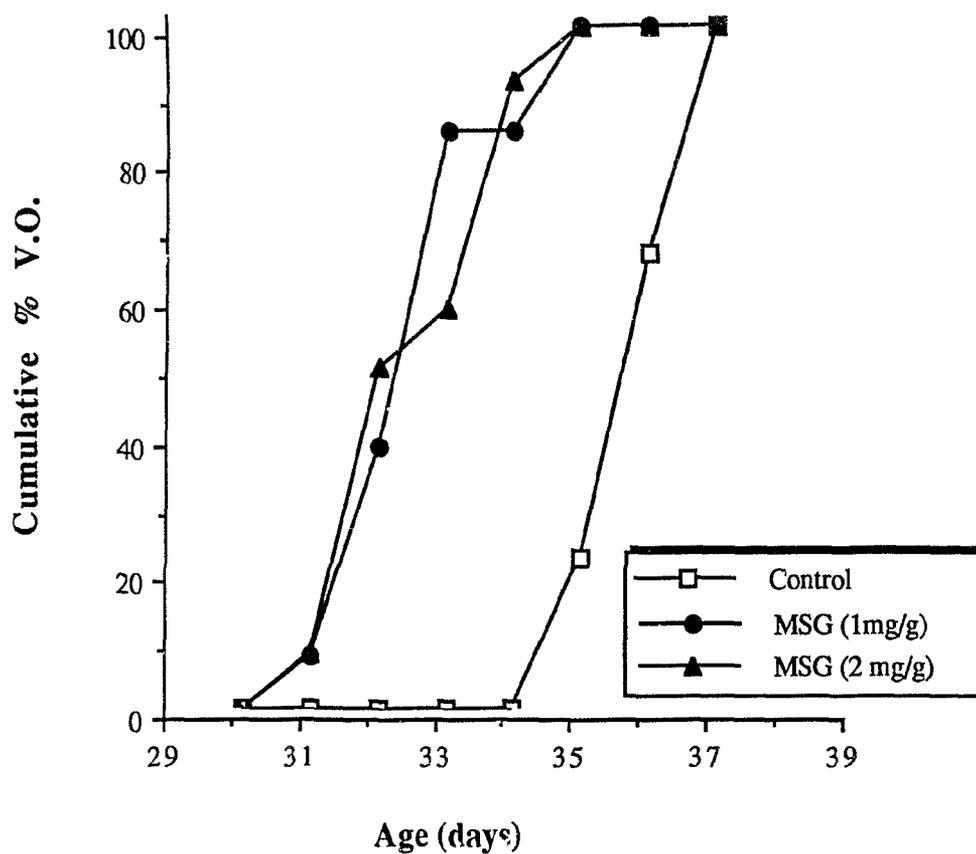
**Fig. 14.** Effect of MSG (4 mg/g body wt.) injected on postnatal days 2 and 4 on the timing of V.O. (n=23). Control rats received 2 injections of saline (n=25). Data plotted as the cumulative % of rats showing V.O.



**Fig. 15.** Effect of MSG (4 mg/g body wt.) injected on postnatal day 2 on the timing of V.O. (n=15). Control rats received 1 injection of saline (n=15). Data plotted as the cumulative % of rats showing V.O.



**Fig. 16.** Effect of neonatal MSG (4 mg/g; days 2 and 4 after birth) on growth rate. Controls received 2 saline injections. Values are mean  $\pm$ s.e.m.

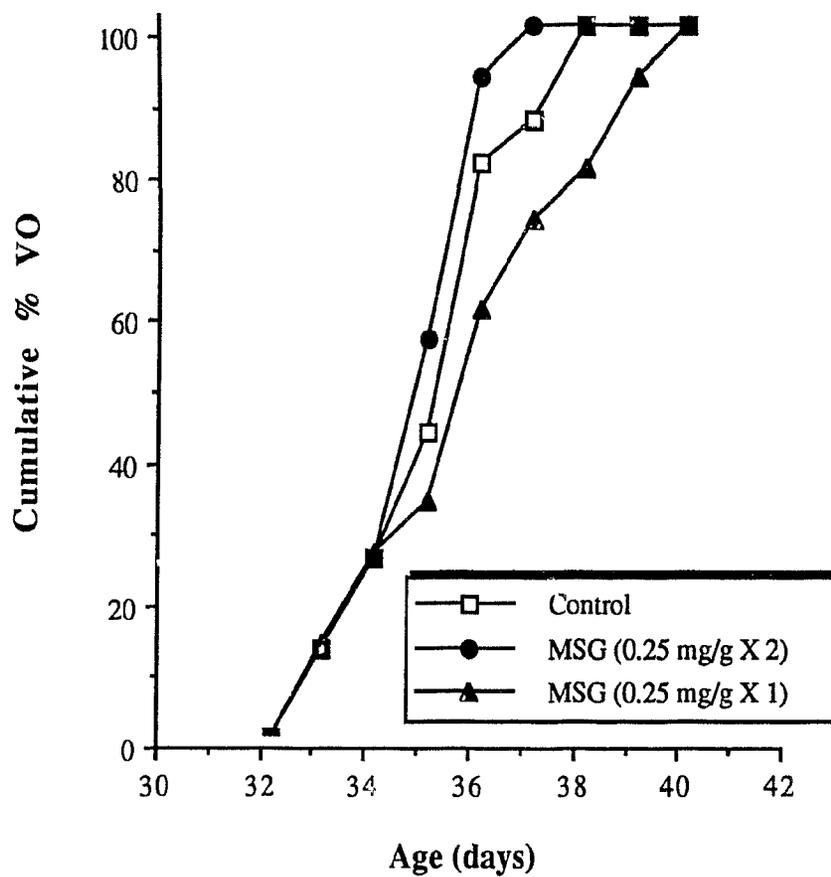


**Fig. 17.** Effect of a single injection of MSG (1 mg/g; n=13 or 2 mg/g; n=12) on postnatal day 2, on the timing of V.O. Control rats (n=10) received a single injection of saline. Data plotted as the cumulative % of rats showing V.O.

**Table 7.** Effects of low-dose neonatal MSG on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

	Control	MSG (1 mg/g; on day 2 )	MSG (2 mg/g; on day 2)
n (rats/group)	10	13	12
Age at V.O. (days)	36.1 $\pm$ 0.2	32.8 $\pm$ 0.3***	32.8 $\pm$ 0.3***
Body Wt. (g)	115.7 $\pm$ 2.1	102.0 $\pm$ 2.1***	96.0 $\pm$ 1.9***
Paired Ovarian Wt. (mg/100 g B.W)	31.8 $\pm$ 1.7	34.3 $\pm$ 2.1	33.1 $\pm$ 1.2
Total CL	11.5 $\pm$ 0.4	11.2 $\pm$ 0.4	10.9 $\pm$ 0.6

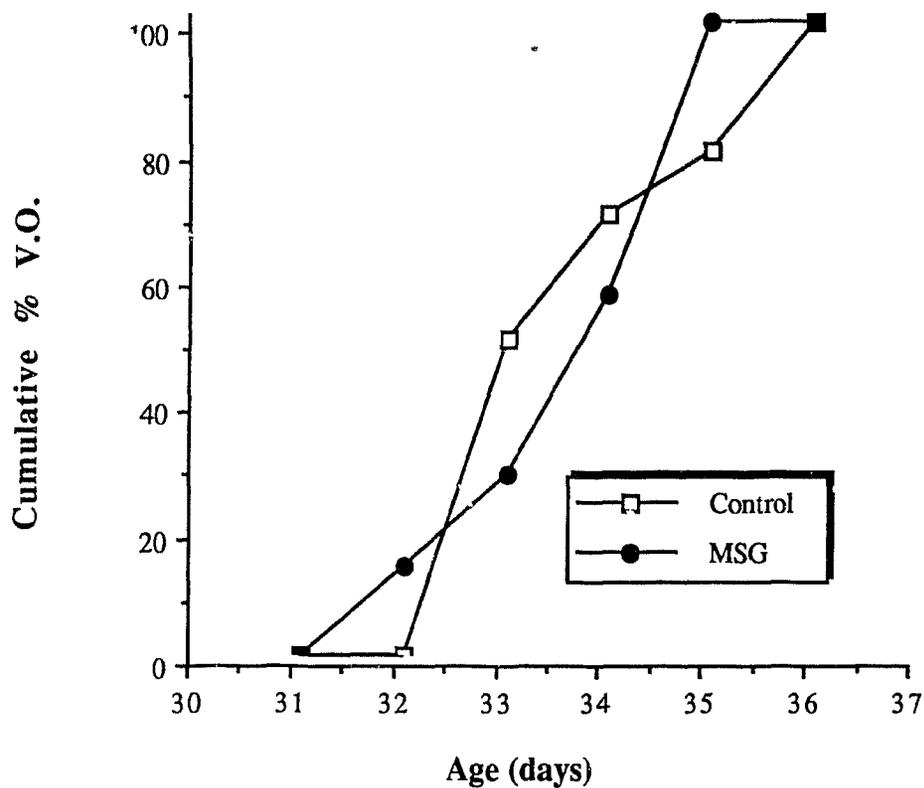
\*\*\* p<0.005 when compared to saline injected controls; ANOVA and post hoc Dunnett t-test.



**Fig. 18.** Effect of MSG (0.25 mg/g body wt.), injected on postnatal days 2 and 4 (n=12) or on postnatal day 2 (n=12) on the timing of V.O. Control rats received 2 saline injections (n=12). Data plotted as the cumulative % of rats showing V.O.

**Table 8.** Effects of ultra-low dose neonatal MSG on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m..

	Control	MSG (0.25 mg/g; on day 2 )	MSG (0.25 mg/g; on day 2 and 4)
n (rats/group)	16	13	17
Age at V.O. (days)	35.5 $\pm$ 0.3	35.6 $\pm$ 0.5	35.1 $\pm$ 0.2
Body Wt. (g)	109.4 $\pm$ 1.0	111.4 $\pm$ 2.1	109.7 $\pm$ 2.1
Paired Ovarian Wt. (mg/100 g B.W)	31.3 $\pm$ 1.1	30.9 $\pm$ 1.4	31.1 $\pm$ 0.9
Total CL	10.5 $\pm$ 0.7	10.0 $\pm$ 0.7	9.7 $\pm$ 0.6



**Fig. 19.** Effect of MSG (4mg/g body wt.) injected on 10 and 12 days postnatally, on the timing of V.O. (n=10). Control rats received 2 injections of saline (n=10). Data plotted as the cumulative % of rats showing V.O.

ovarian weights and number of C.L.s different when compared to controls (Table 9).

#### 9. Effect of Neonatal MK-801 and MSG on Sexual Maturation

The toxic effect of MSG (4 mg/g; days 2 and 4) in neonatal rats was blocked by preinjection with MK-801 (1 mg/kg). Figure 20 illustrates that the effect of MSG injected neonatally can be reversed by preinjection of MK-801 (1.0 mg/kg) whereas MK-801 alone (days 2 and 4) had no effect on sexual maturation. The mean age and body weight at V.O. of the MSG-treated rats were significantly less when compared to controls, whereas the MK-801+MSG and MK-801 treated animals were not different from controls (Table 10). A further control group, receiving MK-801 alone (1 mg/kg) on postnatal days 2, 3, 4 and 5 did not survive. Repeated injections of MK-801 at this dose are clearly inadvisable.

#### 10. Time Course of the LH Response to NMDA

Figure 21 illustrates that the NMDA-induced rise in serum LH secretion is maximal approximately 7.5 minutes following NMDA injection. This result is identical to earlier studies using male rats (Schanker and Cicero 1980). LH secretion was still elevated 15 min after injection but returned to basal levels by 30 min.

#### 11. Effect of Different NMDA Doses on LH Secretion

The effect of different doses of NMDA on serum LH are shown in Fig. 22. The dose required to produce half maximal stimulation of LH release (ED<sub>50</sub>) was approximately 3.75 mg/kg, again almost identical

**Table 9.** Effects of MSG (4 mg/g) injected on postnatal days 10 and 12 on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

	Control	MSG (4 mg/g; days 10 and 12)
n (rats/group)	10	10
Age at V.O. (days)	34.1 $\pm$ 0.4	34.0 $\pm$ 0.4
Body Wt. (g)	113.8 $\pm$ 3.3	108.0 $\pm$ 2.9
Paired Ovarian Wt. (mg/100 g B.W.)	30.3 $\pm$ 0.7	30.8 $\pm$ 1.7
Total CL	9.8 $\pm$ 0.5	10.8 $\pm$ 0.6

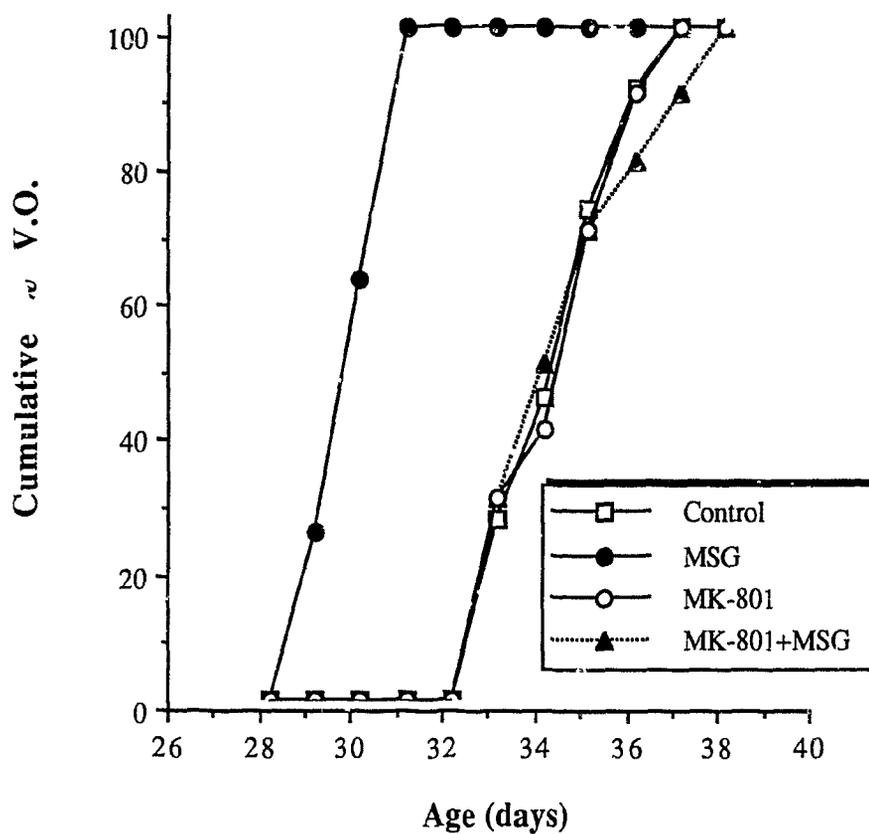
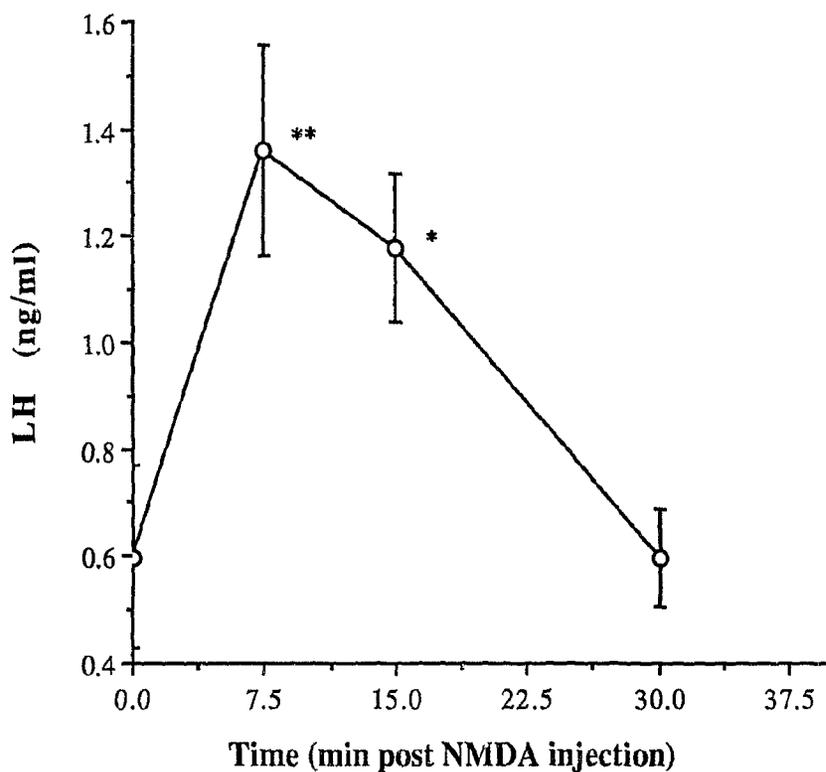


Fig. 20. The ability of MK-801 (1 mg/kg) to block the stimulatory effect of MSG (4 mg/g; days 2 and 4) on the timing of V.O. MK-801 alone had no effect. Data plotted as the cumulative % of rats showing V.O.

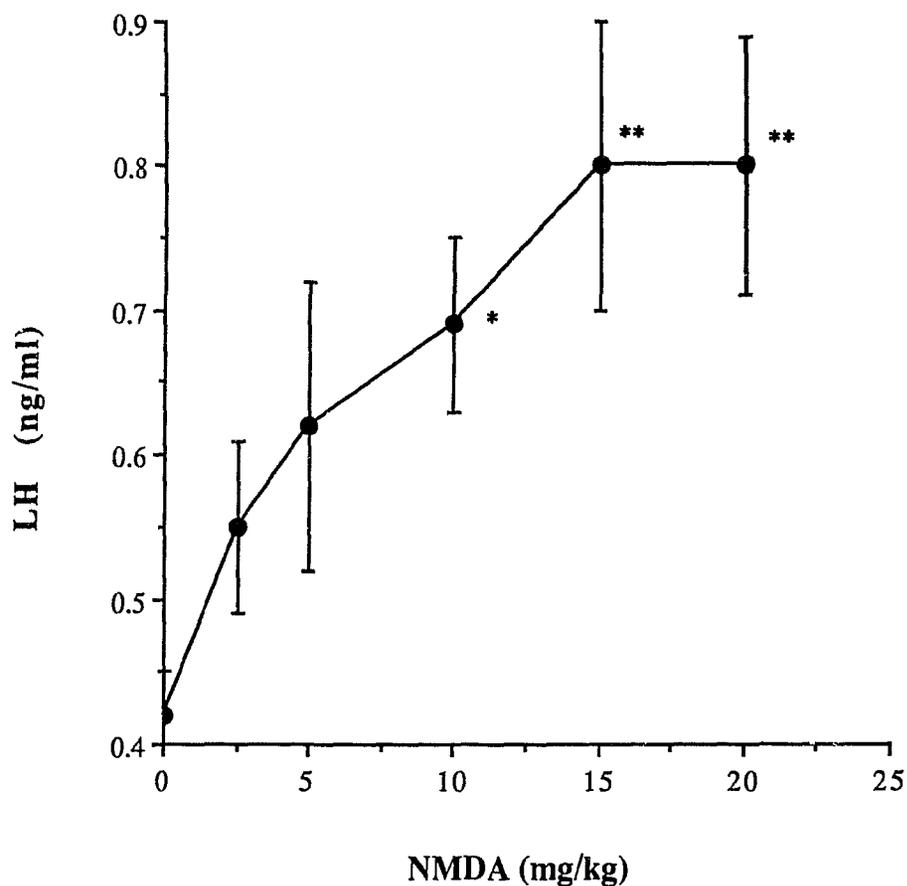
**Table 10.** Effects of neonatal MSG and MK-801 on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean±s.e.m.

	Control	MSG (4 mg/g; days 2 and 4 )	MK-801 (1 mg/kg)	MK-801+MSG
n (rats/group)	11	8	10	10
Age at v.O. (days)	34.6±0.4	30.5±0.5***	34.6±0.5	34.8±0.5
Body Wt. (g)	125.3±2.2	90.8±1.7***	122.1±1.2	119.7±3.2
Paired Ovarian Wt. (mg/100 mg B.W.)	33.7±1.9	34.4±1.3	30.1±1.6	31.1±3.0
Total CL	10.8±0.8	10.2±0.6	10.6±0.3	11.5±0.5

Female neonatal rat pups were injected with MSG ; preinjected with MK-801 followed by MSG ; MK-801 alone or saline on postnatal days 2 and 4. \*\*\* p<0.005 when compared to saline injected controls; ANOVA and post hoc Dunnett t-test



**Fig. 21.** Effect of NMDA (15 mg/kg) on LH secretion in prepubertal female rats (postnatal day 30;  $n=10$ /time point) 7.5, 15, and 30 min after injection.  $*p<0.05$ ;  $**p<0.01$  when compared to saline-injected controls (0 min); ANOVA and post hoc Dunnett t-test Values are  $\pm$ s.e.m.



**Fig. 22.** Effect of different doses of NMDA (2.5, 5, 10, 15, and 20 mg/kg) on LH secretion in prepubertal female rats (post-natal day 30;  $n=10$ /dose). Animals were sacrificed 8 min after NMDA injection. \* $p<0.05$ ; \*\* $p<0.025$  when compared to saline-injected controls; ANOVA and post hoc Dunnett t-test. Values are mean  $\pm$  s.e.m.

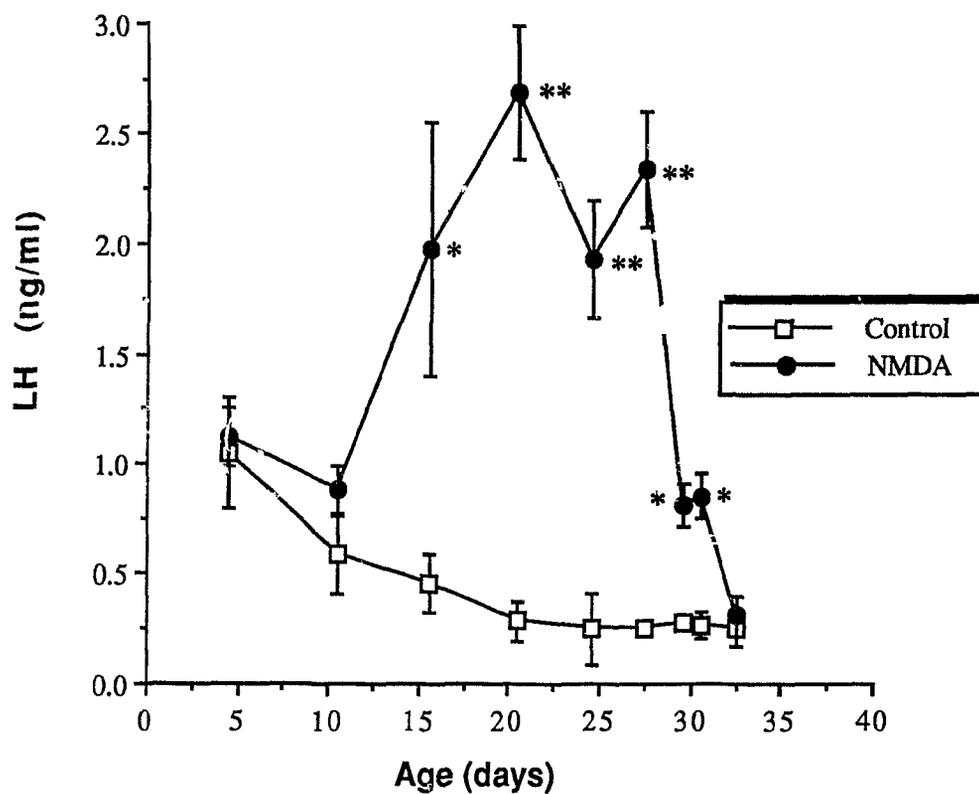
to that previously reported in the prepubertal male (Cicero et al. 1988a). However, the dose of NMDA required to produce maximal LH secretion in the female was between 10-15 mg/kg, almost half the dose required to produce maximal LH secretion in male rats (Cicero et al. 1988a).

### 12. Ontogeny of the LH Response to NMDA

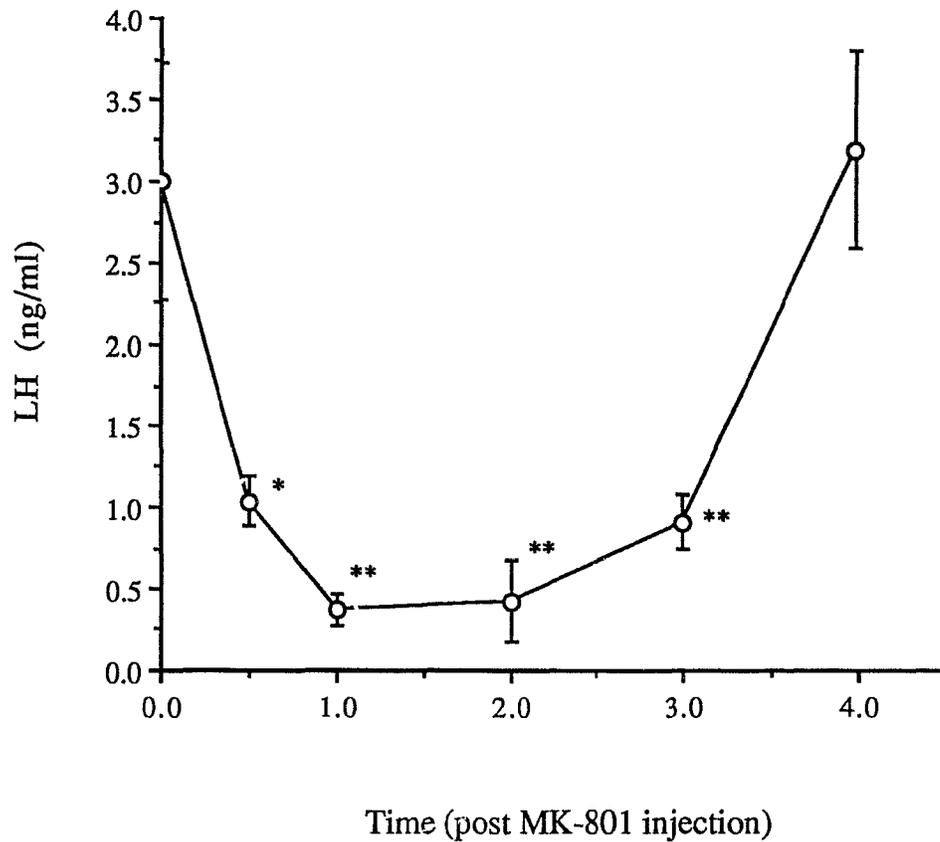
Fig. 23 demonstrates the effect of age on the NMDA-induced increase in serum LH. NMDA (15 mg/kg) was ineffective at increasing LH in 4- and 10-day-old female rats. At postnatal day 15, NMDA elicited a significant increase in LH secretion; peak effectiveness of NMDA was observed between days 15 and 27. On days 29 and 30, the ability of NMDA to elicit an LH response was reduced and by day 32 the NMDA-induced increase in LH secretion was no longer significant when compared to controls. Rat pups between the ages of day 4 and 15 exhibited increased locomotion and tail wagging approximately 6-7 minutes after NMDA injection (just before sacrifice) and 4 pups not sacrificed for trunk blood experienced convulsions 20-30 min later.

### 13. Effect of MK-801 on LH Secretion in Ovariectomized Rats

In earlier experiments (see Fig. 6) MK-801 treatment (0.1mg/kg) of immature female rats was shown to effectively delay V.O. The effect of MK-801 on LH release was investigated in juvenile (day 28) female rats which were ovariectomized and injected 48 hr later with MK-801 (0.1 mg/kg). LH secretion was inhibited. As shown in Fig. 24, MK-801 significantly reduced LH by 30 min post-injection. Minimal



**Fig. 23.** Effect of age on NMDA (15 mg/kg)-induced LH secretion in female rats (n=10-15/group). Animals were sacrificed 8 min after NMDA or saline injection. \*p<0.05; \*\*p<0.001 when compared to saline-injected controls; Student's t-test. Values are mean±s.e.m.



**Fig. 24.** Effect of MK-801 (0.1 mg/kg) on the ovariectomy-induced increase in LH secretion. MK-801 was injected 48 hr after ovariectomy and LH measured over a 4 hr period post-MK-801 injection (n=8/group). \*p<0.01; \*\*p<0.005 when compared to saline-injected controls; ANOVA and post hoc Dunnett t-test. Values are mean±s.e.m.

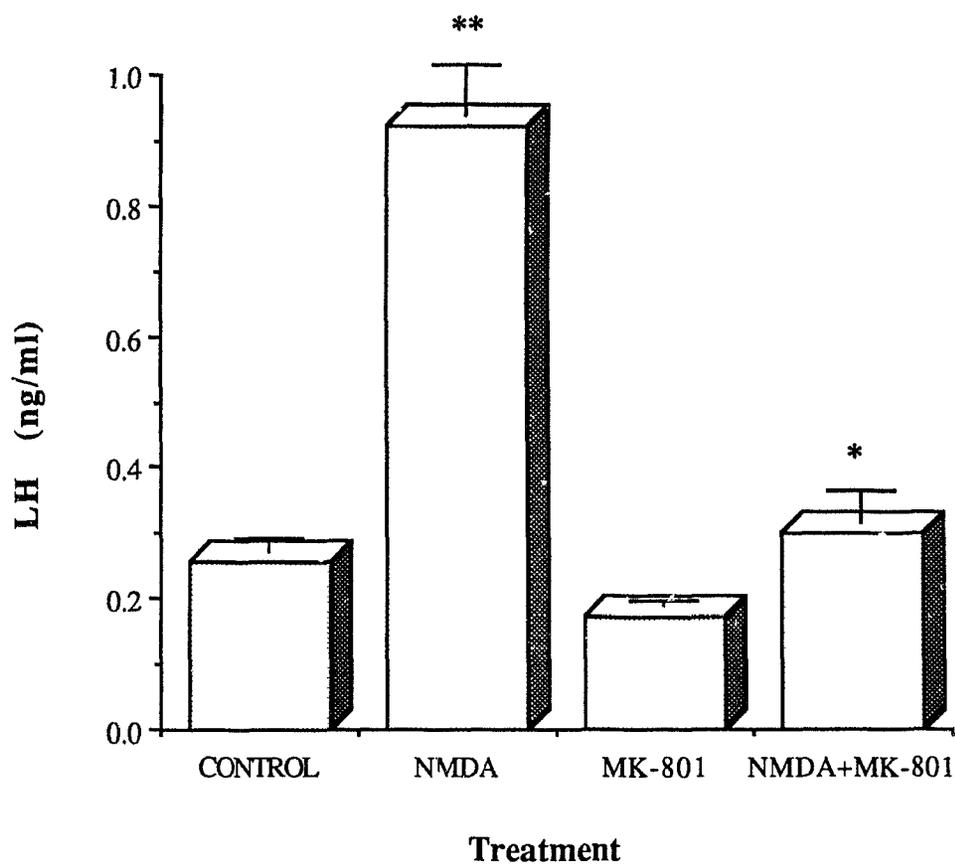
LH values were seen at 1 and 2 hr. LH levels began to increase between 2 and 3 hr following MK-801 injection and returned to control values 4 hr post-injection.

#### 14. Effect of MK-801 on the LH Response to NMDA

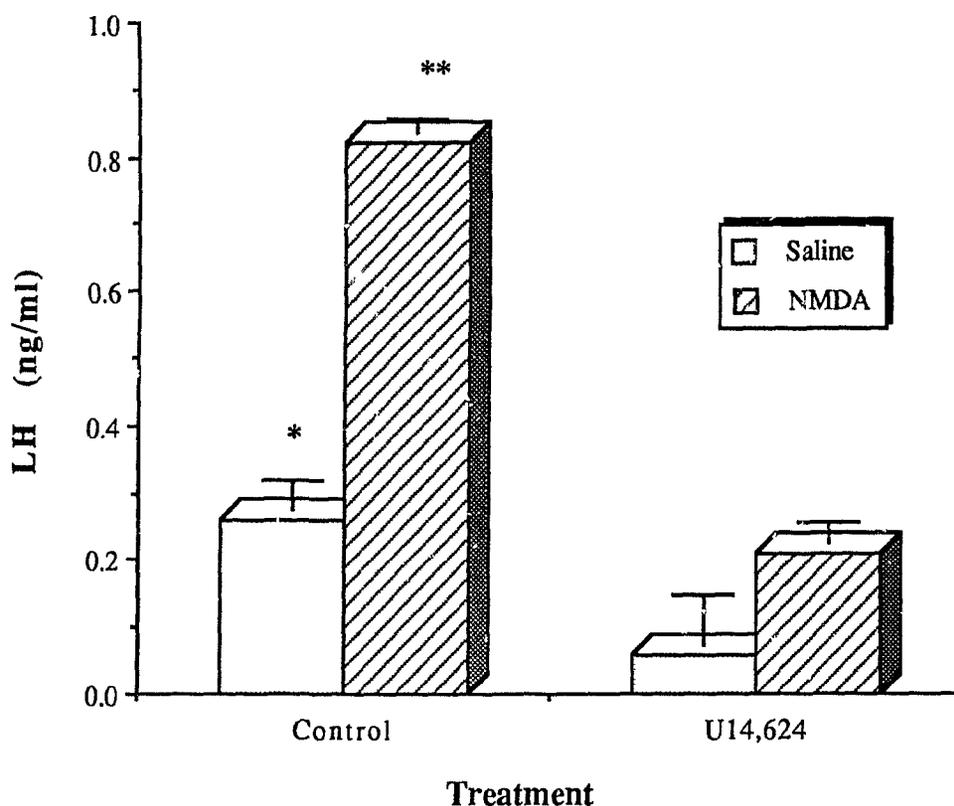
The ability of MK-801 to block the NMDA-induced surge of LH was also examined (Fig. 25). Rats (day 30) preinjected with MK-801 (0.1 mg/kg), followed by NMDA (15 mg/kg) 1 hr later, failed to demonstrate an increase in serum LH, measured at 7.5 min., when compared to NMDA-injected rats (groups of 10 rats; Saline+Saline:  $0.25 \pm 0.02$ ; NMDA+MK-801:  $0.30 \pm 0.05$  ng/ml; vs NMDA:  $1.92 \pm 0.08$  ng/ml;  $p < 0.001$ ) and were almost identical to saline-injected controls. An effect of MK-801 alone was not detectable when compared to controls. This implies that the intact immature female rat is less sensitive to a single injection of MK-801 than is the ovariectomized rat (see Fig. 24).

#### 15. Effect of U14,624 on the LH Response to NMDA

In order to better understand the mechanism by which NMDA stimulates LH release, rats (day 30) were preinjected with the norepinephrine synthesis inhibitor U14,624 (200 mg/kg; i.p.) followed by an NMDA (15 mg/kg) or saline injection 2 hr later. Fig. 26 illustrates that U14,624 pretreatment completely inhibited the NMDA-induced increase in serum LH whereas saline preinjection had no effect (U14624+NMDA:  $0.21 \pm 0.03$  ng/ml,  $n=10$ ; Saline+NMDA:  $0.82 \pm 0.07$  ng/ml,  $n=10$ ;  $p < 0.001$ ). Treatment with U14624 alone significantly decreased serum LH levels (Saline+saline:  $0.26 \pm 0.04$



**Fig. 25.** Effect of MK-801 (0.1 mg/kg) pretreatment (1 hr before NMDA injection) on the NMDA (15 mg/kg)-induced increase in LH secretion in prepubertal female rats ( $n=10$ /treatment). Controls received saline injections. \* $p<0.05$  when compared to MK-801 alone-injected group; \*\* $p<0.001$  when compared to saline injected controls; MK-801-injected rats and NMDA + MK-801 injected rats. ANOVA and post hoc Dunnett t-test. Values are mean $\pm$ s.e.m.



**Fig. 26.** Effect of U14,624 (200 mg/kg i.p.) pretreatment (2 hr) on the NMDA (15 mg/kg s.c.)-induced increase in LH secretion in prepubertal female rats (postnatal day 30; n=10/treatment). U14,624 and saline pretreated controls were injected with either NMDA or saline and sacrificed 8 min later. \* $p < 0.05$  when compared to saline-injected U14,624 pretreated rats. \*\* $p < 0.001$  when compared to saline-injected saline pretreated rats; saline-injected U14,624 pretreated rats; and NMDA-injected U14,624 pretreated rats. ANOVA and post hoc Dunnett t-test. Values are mean  $\pm$  s.e.m.

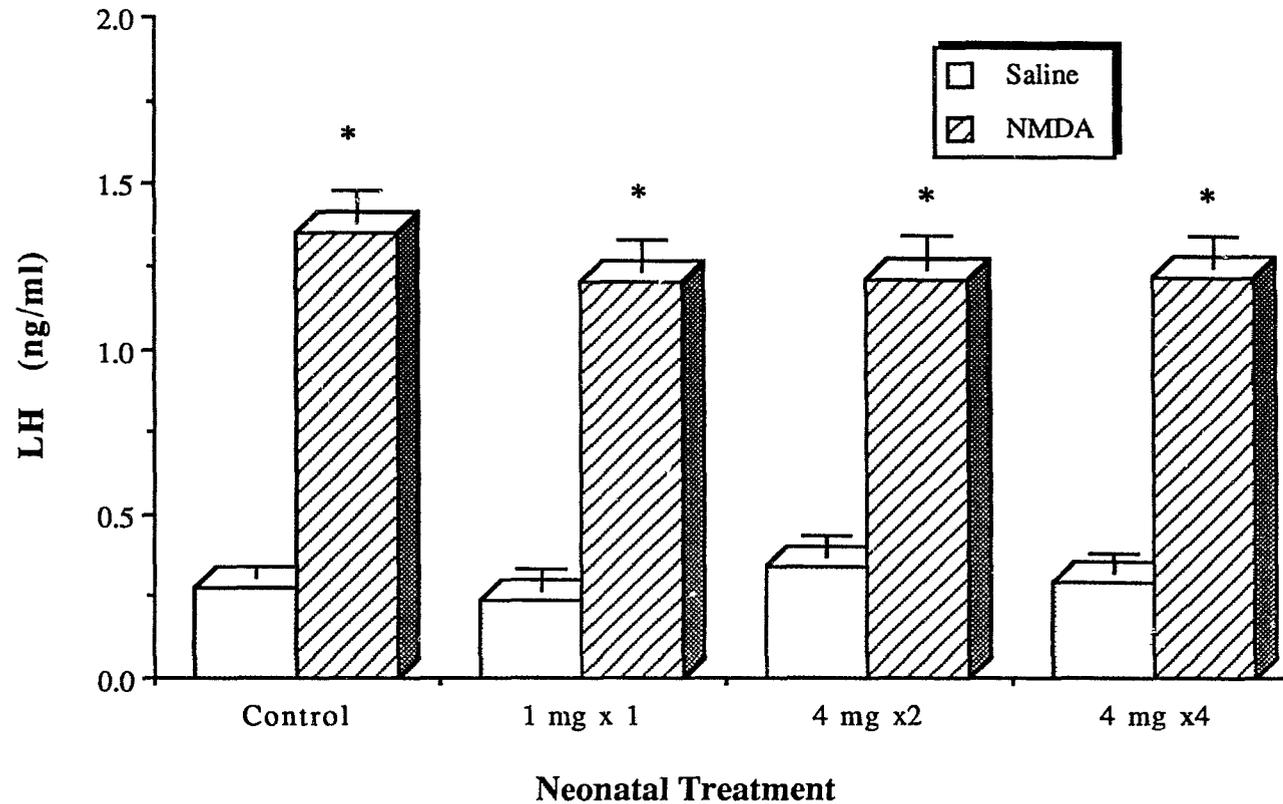
ng/ml, n=10; U14624+saline:  $0.06 \pm 0.02$  ng/ml, n=10;  $p < 0.05$ ) when compared to saline pretreated controls. LH secretion did not differ significantly between U14,624-pretreated animals when injected with saline or NMDA.

#### 16. Effect of Neonatal MSG on the LH Response to NMDA

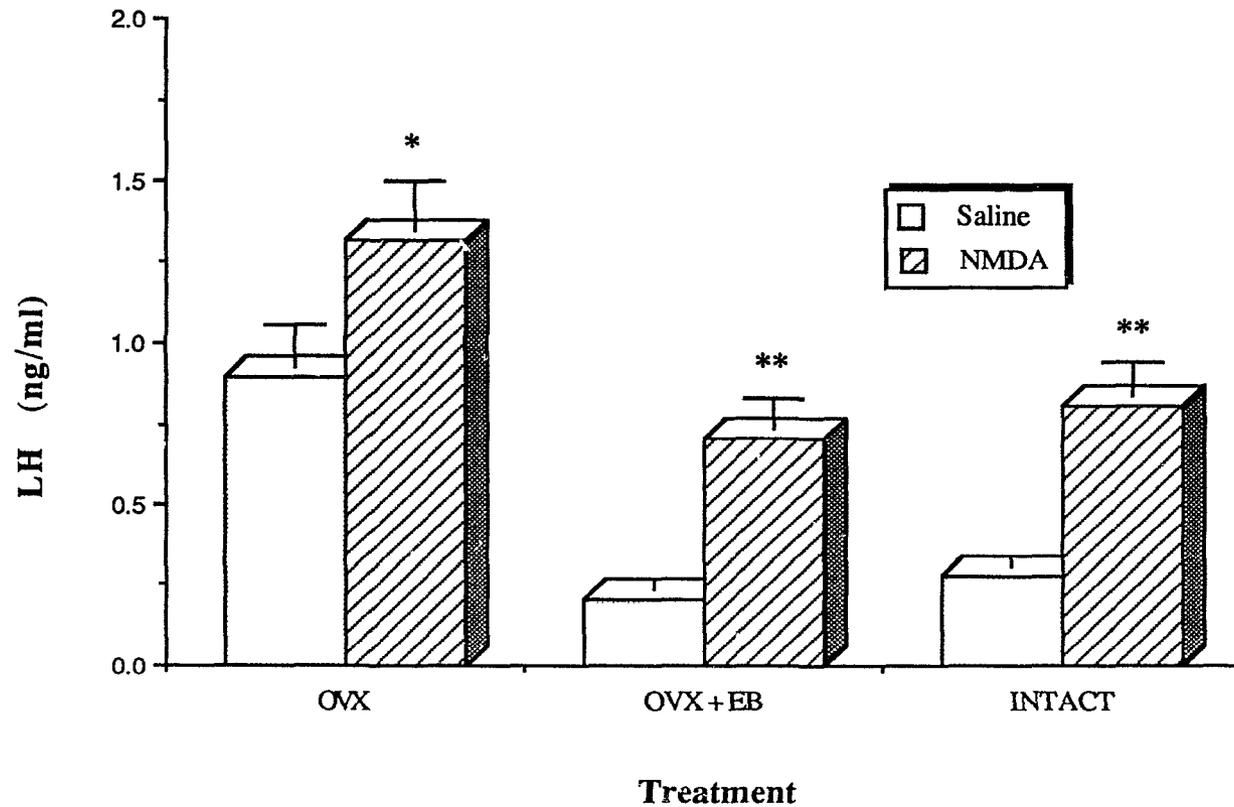
Fernale rats treated neonatally with one of three doses of MSG, all responded with increased LH secretion when injected with NMDA on postnatal day 25 (Fig. 27). The NMDA-induced increase in LH secretion in MSG-treated rats was almost identical to that observed in saline-treated controls. Basal LH levels in MSG-treated controls were also not significantly different from saline-treated controls.

#### 17. Effect of Ovariectomy on the LH Response to NMDA

Earlier experiments (Fig. 24) demonstrated that a single injection of the NMDA antagonist, MK-801, significantly reduced LH secretion in ovariectomized (OVX) rats. Fig. 28 indicates that NMDA increased LH secretion by 45% in rats (postnatal day 30) whose basal LH levels had already been increased as a result of OVX 48 hr earlier (OVX+Saline:  $0.9 \pm 0.12$  vs OVX+NMDA:  $1.3 \pm 0.15$ , n=10/group;  $p < 0.025$ ). When OVX rats received estradiol benzoate (EB; 10  $\mu$ g) on the day of OVX, serum LH concentrations returned to intact control LH levels as a result of steroid negative feedback, when measured 48 hr post-OVX. However, when OVX-EB replaced rats were injected with NMDA 48 hr after OVX, serum LH concentrations increased by 238%, (OVX-EB+Saline:  $0.21 \pm 0.03$  vs OVX-EB+NMDA:  $0.71 \pm 0.09$ , n=10/group;  $p < 0.001$ ) and was comparable to the increase (190%)



**Fig. 27.** Effect of neonatal MSG treatment (1 mg/g on day 2; 4 mg/g on days 2 and 4; 4 mg/g on days 2, 4, 6, and 8; or saline) on the NMDA-induced increase in LH secretion in prepubertal rats (postnatal day 25; 8-10/treatment). Rats from the different treatment groups (MSG or saline) were sacrificed 8 min after injection with either saline or NMDA (15 mg/kg). \* $p < 0.001$  when compared to respective controls. ANOVA and post hoc Dunnett t-test. Values are mean  $\pm$  s.e.m.

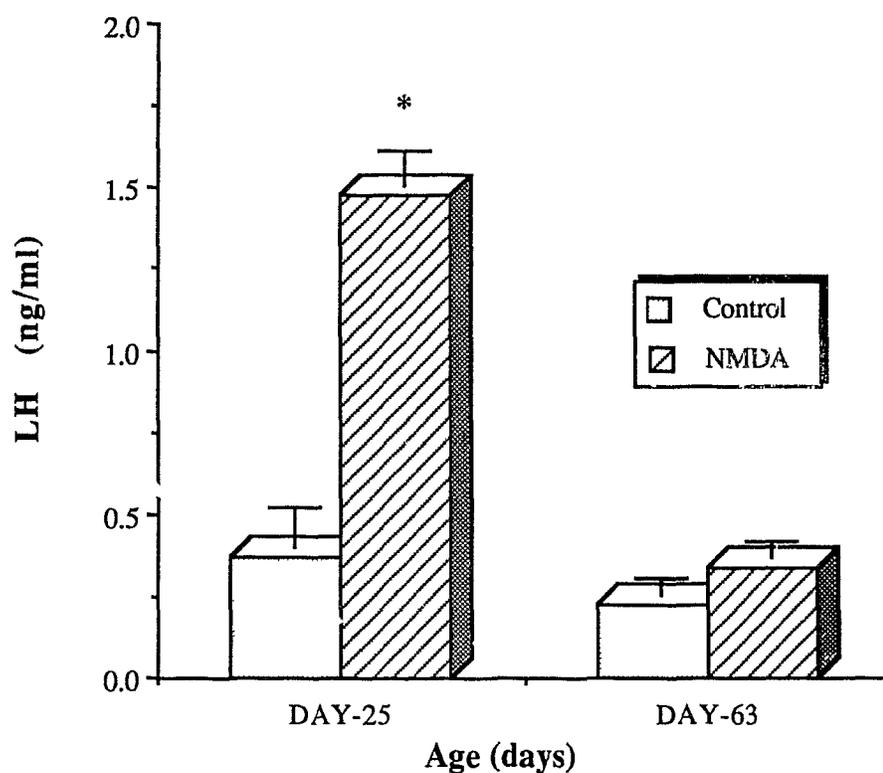


**Fig. 28.** Effect of ovariectomy (OVX) and estradiol benzoate (EB; 10 $\mu$ g; in 0.1 ml sesame oil) replacement on the NMDA (15 mg/kg)-induced increase in LH secretion in prepubertal rats (postnatal day 30; n=10/treatment). Animals were injected with NMDA or saline 48 hr after OVX and sacrificed 8 min later. EB was injected on the day of OVX. \*p<0.025; \*\*p<0.005; \*\*\*p<0.001 when compared to respective controls; ANOVA and post hoc Dunnett t-test Values are mean $\pm$ s.e.m.

observed in NMDA-injected intact rats (Intact+Saline:  $0.28 \pm 0.03$  vs Intact+NMDA:  $0.810 \pm 0.10$ ,  $n=10/\text{group}$ ;  $p < 0.001$ ).

#### 18. Effect of Age on the LH Response to NMDA in the Male Rat

The results from a previous study (Cicero et al. 1988a) that demonstrated an attenuation of the NMDA-induced LH response with increasing age in male rats were also confirmed. As shown in Fig. 29, prepubertal male rats (postnatal day 25) responded to NMDA (15 mg/kg) with a 3-fold increase in LH levels (Control:  $0.37 \pm 0.12$  ng/ml;  $n=6$  vs NMDA:  $1.48 \pm 0.10$  ng/ml;  $n=6$ ;  $p < 0.005$ ) whereas day 63 males were unresponsive (Control:  $0.22 \pm 0.04$  ng/ml;  $n=6$  vs NMDA:  $0.33 \pm 0.05$  ng/ml;  $n=6$ ).



**Fig. 29.** Effect of NMDA (15 mg/kg) on LH secretion in prepubertal (postnatal day 25;  $n=6$ /treatment) and adult (postnatal day 63;  $n=6$ /treatment) male rats. Animals were sacrificed 8 min after injection of NMDA or saline;  $*p<0.01$  when compared to control; ANOVA and post hoc Dunnett t-test. Values are mean $\pm$ s.e.m.

## Discussion

The stimulatory effect of NMDA on GnRH/LH secretion is now well-documented in the rat and the rhesus monkey (Plant et al. 1989; Urbanski and Ojeda 1987). However, only recently have data appeared which demonstrate the excitatory neurotransmitter glutamate to be an endogenous regulator of GnRH secretion (Arslan et al. 1988; Bourguignon et al. 1989). The results reported here, now suggest that glutamate may be a regulatory component of the developmental neural process which culminates in first ovulation in the female rat. The glutamate antagonists dextrorphan and MK-801 are regarded as non-competitive i.e. they prevent the effect of the endogenous neurotransmitter (glutamate) by acting at a site close to, but separate from, that of the agonist (Kemp et al. 1987). The effectiveness of blockade is use-dependent and therefore increases in the presence of glutamate (Wroblewski and Danysz 1989). Thus, drug treatment (day 27 after birth) began at a time in our colony when afternoon pulses of LH should begin (Ojeda and Urbanski 1988). A single daily injection, given at 11:00 hr, of either antagonist significantly delayed first ovulation (Figs. 5 & 6). When the time of MK-801 injections was changed from 11:00 hr to 09:00 hr, the timing of V.O. was unaffected, whereas injections given at 14:30 hr produced the largest delay in sexual maturation of all the injection schedules employed (Fig. 8). These results suggest that MK-801 treatment given early in the light period (09:00 hr), does not provide an effective blockade of afternoon LH pulses and V.O. occurs normally. Conversely, MK-801 injections between 11:00 hr and 14:30

hr effectively delay first ovulation possibly by antagonizing NMDA receptor mediated LH secretion. The observation that MK-801 injected from postnatal day 30 until V.O. is ineffective at delaying V.O. (Fig. 9) suggests that the neural development and processes that modulate LH pulsatility from day 27 onward, are critical for the events that culminate in first ovulation. Thus, rats may become resistant to MK-801 blockade imposed from day 30 until V.O. as a result of the neural organization that occurs between day 27 and day 29. It is obvious that this limited time frame is important in the NMDA (glutamate)-mediated events that lead to first ovulation and therefore was examined in terms of ontogenesis of NMDA-induced LH secretion (see below). The competitive NMDA antagonist APV, however, did not induce a delay in sexual maturation when injected from day 27 until V.O. (Fig. 7). This may be due to the low dose of APV (1 mg/kg) used to block GnRH/LH pulsatility. This suggestion is supported by experiments performed by Urbanski and Ojeda (1990) who demonstrated that at least 4 intravenous (i.v.) injections of APV (30 mg/rat; between 11:30 hr and 17:30 hr) were required to block the estradiol-induced midafternoon surge of LH in ovariectomized rats. Conversely, only a single injection (s.c.) of MK-801 (0.2 mg/kg; at 11:30 hr) was required to block the same LH surge. Similar experiments in which ovariectomized prepubertal rats were treated with MK-801 (0.1 mg/kg) have been performed and are discussed below.

There is no ready explanation to account for the eventual occurrence of first ovulation which takes place in the face of continued non-competitive antagonist treatment. All of the

dextrorphan- and 56% of the MK-801-tested rats ovulated (Table 1). The daily dose of antagonist drug might allow the emergence of a supersensitive response to endogenous glutamate. In electrophysiological terms, recovery from blockade appears to be accelerated by repeated stimulation with glutamate (Kemp et al. 1987). Alternatively, non-NMDA sensitive glutamate receptors may become important as puberty approaches (Lopez et al. 1990; Donoso et al. 1990)

Preliminary experiments examining the influence of MK-801 in adult, cycling female rats show that in this model glutamate receptors may also be important for reproductive cyclicity. However, it seems crucial to employ a low dose of MK-801 to avoid its obvious toxicity.

In experiments in which puberty was induced by PMS, MK-801 was able to block the expected ovulation at a dose of 1 mg/kg but not at 0.1 mg/kg. A single injection of MK-801 (1 mg/kg), given in the afternoon of presumptive proestrus, was also sufficient at preventing ovulation by arresting the PMS-induced stimulation at the stage of proestrus. A similar group of rats ovulated 24 hr later i.e. without further MK-801 treatment, a result which suggests that the influence of MK-801 is readily reversible. This observation is not unlike the consequence of barbiturate anaesthesia in cycling rats, described in the classic work of Everett and Sawyer (1950). These results, based upon a simple bioassay (ovulation), suggest that gonadotropin secretion is compromised by MK-801. This has been examined in more detail below.

Previously published work by Urbanski and Ojeda (1987) demonstrated that frequent and repetitive stimulation of immature female rats with N-methyl-D,L-aspartic acid (NMA; 4 days; 30 min pulses during 1300 to 1700 hr each day) induces precocious sexual maturation. This observation is consistent with results of similar experiments in which pulsatile GnRH treatment advanced the timing of V.O. (Urbanski and Ojeda 1987). It was therefore surprising, in the present experiments, to observe that a fraction of the NMA dose used by Urbanski and Ojeda (1987) (in terms of NMDA, which was used in the experiments reported here), was sufficient to produce precocious first ovulation in our colony (Fig. 10). Inspection of the data in Fig. 11 indicates that single injections of NMDA (15 mg/kg) appear to synchronize the timing of V.O. and first ovulation. This phenomenon needs to be examined in more detail though it concurs with the general theme of this section that hypothalamic glutamate receptors exert some control over the neural events which culminate in first ovulation.

The delaying effect of MK-801 on first ovulation was inhibited by preinjection of NMDA. This result suggests that the effect of MK-801, which binds to the site within the cation channel of the receptor, can be reversed by NMDA binding to the NMDA-specific glutamate receptor subtype. The anterior pituitary is non-responsive to NMDA (Schanker and Cicero 1980; Tal et al. 1983) and so NMDA-sensitive neurons are very likely to be located in the circumventricular organs, such as the median eminence, which lie outside the blood-brain barrier. Interestingly, recent work by Ondo et al. (1988) demonstrates that the medial preoptic area (MPO) and not the

arcuate/median eminence, is the site of action of NMDA in the rat. In contrast, Bourguignon and his associates (1989) observe glutamate-induced secretion of GnRH from hypothalamic fragments (albeit with high concentrations) which do not contain the MPO. This is an interesting problem which deserves further study. Note, however that both these groups used older, post-pubertal male rats which are not particularly sensitive to NMDA (Cicero et al. 1988a).

The marked influence of neonatal MSG treatment on the precocious timing of first ovulation (Figs. 13-19) was prevented by co-injection of MK-801 (Fig. 20). This result demonstrates that the effects of MSG are mediated through an NMDA-type receptor. We can also suggest that MSG, an axon sparing neurotoxin, eliminates the majority of arcuate neurons which possess glutamate receptors (Rothman and Olney 1987). In fact there is evidence that MSG-lesioned rats are unable to respond to NMDA with release of LH when tested at day 25 of life (Olney and Price 1980). The implication here is that an intact arcuate nucleus is required for a normal response to NMDA. The results of similar experiments presented in this Thesis are discussed below. The question remains, how do the arcuate-lesioned female rats reach puberty precociously? The observations reported here are reminiscent of the seminal work of Donovan and Van der Werff Ten Bosch (1965) who demonstrated that electrolytic lesions of the anterior hypothalamus in newborn rat pups induced precocious first ovulation several weeks later. The logical explanation for this result was to postulate that sexual maturation is accelerated subsequent to the removal of some type of hypothalamic restraint. This appears to be a reasonable interpretation of our own data. Three cell types

which are largely destroyed by MSG i.e. those containing dopamine (DA), pro-opiomelanocortin-derived peptides (e.g.  $\beta$ -endorphin) and GABA, are thought to negatively control gonadotrophin secretion (Bicknell 1985; Masotto and Negro-Vilar 1987). Neurons which contain norepinephrine, serotonin and GnRH are spared (Jennes et al. 1984). However, estradiol target neurons are also eliminated by MSG (Jennes et al. 1984) and dopamine (Nemeroff 1984),  $\beta$ -END (Alessi et al. 1988) and GABA (Jennes et al. 1984) concentrations are all severely reduced in MSG-treated rats. These neural deficits are accompanied by profound endocrine abnormalities (Nemeroff 1984; Olney and Price 1983).

The use of the medium-dose of neonatal MSG (X2 injections; 4 mg/g body weight), in agreement with Dawson (1986), had little influence on normal prepubertal growth (Fig. 16). More recent studies by Dawson et al. (1989), using MSG injection schedules of 4 mg/g on days 2 and 4 or days 2, 4, 6, and 8, reported no significant alterations in the day of V.O. or early estrous cyclicity. In contrast, the results presented here demonstrate that neonatal MSG treatment, using doses ranging from as high as 4 mg/g (X4) to as low as 1 mg/g (X1), will significantly advance the mean day of V.O. and at the higher doses, disrupt estrous cyclicity. Only at doses of 0.25 mg/g (X1 or X2) or 4 mg/g on postnatal day 10 and 12 was MSG ineffective on the timing of sexual maturation (Figs. 18 & 19).

The data presented here, demonstrate that hypothalamic glutamate receptors, possibly of the NMDA-subtype, comprise a significant regulatory component of the neural control of sexual maturation. Stimulation of neonatal glutamate receptors with MSG appears to

eliminate some type of hypothalamic restraint mechanism, which culminates in precocious first ovulation. In keeping with previous work (Wilkinson and Landymore 1989) we can suggest that opioid neurons may be one essential component of this restraint. Wiemann and co-workers (1989) have recently demonstrated that neurons in the rostral arcuate nucleus contain POMC mRNA which appears to be sensitive to gonadal steroids. It is this part of the arcuate nucleus which is destroyed by MSG (Seress 1982), which would therefore account for the observed deficit of  $\beta$ -END (Alessi et al. 1988). Alternatively, there is evidence implicating MSG as a neuronal growth factor, possibly influencing neuronal differentiation (Aruffo et al. 1987). Thus one can envisage a reorganization of the glutamate innervation of the hypothalamus, subsequent to a MSG lesion, as has been proposed by Rose and Weick (1987) in adult rats.

Although a few studies have looked specifically at the effects of glutamate agonist and antagonist treatment on sexual maturation (Urbanski and Ojeda 1987; Plant et al. 1989; Urbanski and Ojeda 1990; this Thesis), the most extensive work has examined the excitatory influences of EAA on LH secretion *in vivo* (Olney et al. 1976; Schainker and Cicero 1980; Cicero et al. 1988a) and GnRH release *in vitro* (Bourguignon et al. 1989a,b). Unfortunately these investigations were performed on male rats and therefore the results obtained may not be applicable when investigating the stimulatory effects of EAA on prepubertal female rats.

The results presented here indicate that maximum LH secretion occurs approximately 7-8 min post-NMDA injection (Fig. 21) and is identical in both male and juvenile female rats. The dose of NMDA

required to elicit maximal LH secretion is two-fold less for females than males and suggests that females are much more sensitive to the stimulatory effects of NMDA on LH secretion. This could be due to differences in NMDA-specific glutamate receptors. Although a number of studies have looked at the ontogenesis of NMDA receptors (Tremblay et al. 1988; Greenamyre et al. 1984; Insel et al. 1990) sex differences in EAA receptors during maturation, especially in the hypothalamus, have received little attention. To obtain a developmental profile of the effectiveness of NMDA in the induction of LH secretion, NMDA was injected (15 mg/kg; s.c.) beginning on postnatal day 4 through to postnatal day 32. The results presented here are similar to those of Cicero et al. (1988a) and demonstrate that LH release is most sensitive to NMDA stimulation from postnatal day 10 to postnatal day 30 (Fig. 23). However, contrary to the observations of Cicero et al. (1988a) in the immature male rat, the ability of NMDA to evoke LH secretion decreased dramatically at day 32, resulting in serum concentrations of LH that were indistinguishable from saline-injected controls. At present it is not known whether the attainment of first ovulation would further modify the LH response to NMDA. In these experiments all of the rats used in the postnatal day 32 group had not reached vaginal opening and had not ovulated. Nevertheless, at this age between 20% and 30% of rats have normally reached V.O. These were eliminated from the experimental group. Clearly, large numbers of rats will be required to determine the response to NMDA in the days and hours immediately preceding first ovulation.

The marked decline in the LH response to NMDA which occurs between postnatal day 30 and day 32 (Fig. 23) may not just be due to a change in receptor sensitivity. Previous studies have shown (Wilkinson and Moger 1981) that the pituitary responsiveness to GnRH decreases dramatically between postnatal day 25 and postnatal day 30. Therefore glutamate and EAA receptor activation could have little influence on LH release at this age even though GnRH secretion may increase. *In vitro* studies on hypothalamic GnRH release, such as those performed with male rats (Bourguignon et al. 1989a,b), would obviously provide a more complete picture during this stage of development. It should also be noted that studies designed to examine the post-pubertal effects of estrous cyclicity on NMDA-induced LH release are important and in need of investigation.

Neural degeneration in the arcuate nucleus is not observed in juvenile rats (postnatal days 20-30) post-NMDA injection (15 mg/kg) (Olney et al. 1976; Price et al. 1978b; Schainker and Cicero 1980). However, female rats 10 days and younger experienced seizures 10-15 min after NMDA treatment and died 30-60 min later. While this suggests that the blood brain barrier may be more permeable to NMDA at these young ages, the resulting NMDA-induced excitotoxicity could be related to the number and sensitivity of EAA receptors. Tremblay et al. (1989) have shown that hippocampal NMDA-sensitive sites (CA1-stratum-radiatum) are greatest at postnatal day 8 and decrease to adult levels by postnatal day 13. This decrease is due to a reduction in receptor number and is not the result of a decrease in receptor affinity. Similar observations have been made in globus pallidus (Greenamyre et al. 1984). Conversely,

autoradiographical studies by Insel et al. (1990), have shown that in different brain regions, NMDA receptor number is maximal between postnatal days 20-30, which corresponds to the time when we see maximal NMDA-evoked LH response. Interestingly, studies by Shinohara et al. (1990) have demonstrated that binding sites for glycine, an allosteric modulator of the NMDA receptor (Johnson and Ascher 1987), do not reach adult levels until postnatal day 10. It is not inconceivable that developmental changes in the number of glycine binding sites could mediate the ontogenetic shifts in NMDA-induced LH release observed during the first two weeks of postnatal life. It is also possible that an increase in kynurenic acid, an endogenous glycine antagonist, during development (Moroni et al. 1988) may be responsible for the attenuated NMDA-induced LH response observed as adulthood approaches.

Suppression of the high post-ovariectomy levels of LH by MK-801 (0.1 mg/kg; Fig. 24) suggests that endogenous glutamate and NMDA receptor activation play an integral role in the regulation of LH secretion. These results demonstrate that the inhibitory influence of MK-801 on LH output are not reversed until 4 hr post-injection. This corresponds with observations that injection of MK-801 at 9:00 am, beginning on postnatal day 27 does not delay vaginal opening (see Fig 8). Note however, that a single injection of MK-801 - at this concentration (0.1 mg/kg) - does not reduce serum LH levels below that of controls (see Fig. 25). This suggests that NMDA receptors may be less sensitive in the intact female rat when compared to those of ovariectomized rats (Fig. 24). Dose response studies need to be performed on intact prepubertal female rats. The possibility that

basal levels of serum LH are maintained by other EAA receptors i.e. kainate, cannot be overlooked and indeed may play a critical role in the modulation of GnRH and LH secretion (Lopez et al. 1990; Donoso et al. 1990).

It is well known that norepinephrine synthesis inhibitor attenuates the release of LH in female rats (Ramirez et al. 1984). NMDA is also reported to regulate norepinephrine release in cerebral cortex (Fink et al. 1989). Results from the present study indicate that pretreatment of immature rats with U-14,624, a drug that inhibits dopamine  $\beta$ -hydroxylase activity, significantly reduces NMDA-induced LH secretion (Fig. 26). This suggests that the NMDA-induced increase in LH secretion does not occur via NMDA receptors located on the GnRH neuron terminal. Rather, NMDA receptor activation probably mediates the release of norepinephrine and subsequent GnRH secretion. This mechanism is not unlike that proposed for naloxone-induced LH secretion (Kalra and Kalra 1983). Note also, that basal levels of serum LH decreased after U14,624 treatment. This suggests that norepinephrine plays an integral role in the maintenance of basal LH secretion. Although not significant, the small NMDA-induced increase in serum LH in animals pretreated with U14,624, suggests that NMDA may mediate LH secretion through a mechanism other than NE release. These data seem to contradict those of Johnson et al. (1985) who reported that the dopamine  $\beta$ -hydroxylase inhibitor FLA-63 did not prevent the stimulation of LH secretion by N-methyl-D,L-aspartate (NMA). However, these latter experiments were performed in post-pubertal female rats injected intracisternally with NMA. The opposite results obtained in these two

studies deserve further attention. Although the actual site of NMDA-regulated GnRH release is unknown, *in vitro* studies (Bourguignon et al. 1989b) have suggested that the mediobasal hypothalamus (arcuate-median eminence) is the primary site of control (but see Ondo et al. 1988). The subfornical organ (SFO), a circumventricular organ like the arcuate-median eminence, has also been implicated as a possible locus for GnRH activation (Donevan et al. 1989). These studies have shown that electrical stimulation of the SFO results in significantly elevated serum LH levels. It is therefore not unreasonable to suggest that stimulation of this area as a result of peripheral injection of NMDA may indirectly elicit GnRH release. These results also suggest that the pathway from SFO stimulation to GnRH release may be mediated by norepinephrine.

The NMDA-induced increase in serum LH in prepubertal female rats neonatally treated with different doses of MSG (Fig. 27) are similar to the result of Badger et al. (1982) using MSG-treated adult male rats. Both of these studies contradict the results of Olney and Price (1980). They reported that male rats neonatally treated with MSG (2-4 mg/g increased over days 2, 4, 6, 8, and 10) did not respond to NMA injection (25 mg/kg; postnatal day 25) with an increase in serum LH concentration. They concluded that because neonatal MSG treatment destroyed cell bodies in the arcuate nucleus possessing glutamate receptors, an NMA-induced LH response would not be expected. In the studies presented here, both control male and female as well as MSG-treated female rats respond to NMDA stimulation with increased serum LH. on postnatal day 25 (Fig. 27 & 29). This suggests that neonatal MSG may be more deleterious to

male rats (i.e. males are more sensitive to neonatal MSG) compared to the female rats used in this study. However, the experiments of Badger et al. (1982) using adult male rats do not support this suggestion.

The NMDA-induced increase in serum LH observed in OVX rats (Fig. 28) is similar to the naloxone-induced LH increase in OVX rats reported by Bhanot and Wilkinson (1983). In both studies the percent increase in naloxone- or NMDA-induced LH secretion was less in OVX rats than in intact. The effect of NMDA in OVX rats reported here is also similar to that in castrated males reported by Schainker and Cicero (1980). That NMDA induced an increase in serum LH in the absence of sex-steroids suggests that this effect is not mediated by the disinhibition of steroid negative feedback on the hypothalamic-pituitary-gonadal axis. Studies using long-term castrates are needed.

However, the difference in percent increase of NMDA-induced LH secretion between OVX and OVX-EB replaced or intact, suggests that estradiol may potentiate NMDA receptor activation (Smith 1989) and increase LH secretion. Such a role for sex-steroids has been demonstrated by Estienne et al. (1990) who observed that estradiol replacement in OVX sheep was required for NMDA to increase LH secretion.

Using NMDA agonists and antagonists and neonatal treatment with MSG, these studies have demonstrated that hypothalamic glutamate receptors - possibly of the NMDA subtype - comprise a significant regulatory component of the neural control of sexual maturation. Experiments presented here have also characterized the stimulatory

effect of NMDA on LH secretion in prepubertal female rats, in an attempt to better define the role of the NMDA receptor in the control of the onset of puberty.

Briefly discussed in this section was the possibility glutamate and opioid peptides could concurrently regulate LH release. Experiments designed to investigate this possibility are described in Section. III Results (p. 170). The results suggest that opioid and glutamate regulation of GnRH/LH secretion is exerted through two distinct but converging pathways.

## Section II. NMDA AND THE INDUCTION OF C-FOS GENE EXPRESSION IN RAT BRAIN

### Introduction

The *c-fos* gene is the normal cellular counterpart of the viral oncogene *v-fos* which causes osteogenic sarcoma (Curran and Morgan 1987). The name *fos* is derived from the first letter of the closely related mouse virus (FBJ and FBR) which produces osteogenic sarcoma, the tumor line from which the last two letters are derived (for review see Curran 1988). Viral oncogenes are copies of the genes that are a normal constituent of the cellular genome. The cellular genes (or proto-oncogenes) from which viral oncogenes arose, seem to be involved in the regulation of cell development, growth and differentiation (Herschman 1989). This is based, in part, on the observations that deregulated expression of *c-fos* in fibroblasts causes unrestricted cell proliferation (Miller et al. 1984) and that a variety of polypeptide growth factors increase the expression of *c-fos* and its 55 kilodalton phosphoprotein product (55 *c-fos*) (Morgan and Curran 1986; Ran et al. 1986).

*C-fos* belongs to a group of genes (i.e. *c-fos*, *c-jun* and *c-myc*) termed cellular immediate early genes (IEG) (Greenberg et al. 1985; Morgan and Curran 1986; Curran and Morgan 1987; Sheng and Greenberg 1990) because gene transcription is activated rapidly and transiently within minutes of stimulation. A second group, the late response genes, whose transcription is induced more slowly (i.e. hours) are dependent on new protein synthesis (Sheng and

Greenberg 1990). The transcription products of IEG (i.e. Fos protein) are thought to control the expression of the late response genes. Recent evidence suggests that the Fos and Jun proteins associate with one another in a heterodimeric nucleoprotein complex that binds with high affinity to the consensus AP-1 (TGACTCA) (transcription factor) binding site (Rauscher et al. 1988a,b; Sonnenberg et al. 1989a). Since the AP-1 regulatory element is essential for transcription of certain genes (Franza et al. 1988; Rauscher et al. 1988a,b) Fos and Jun may be an important link between cell stimulation and subsequent alteration in gene expression (Curran and Morgan 1987).

The pheochromocytoma cell line (PC12) has been used extensively to study the role of IEG such as c-fos in the nervous system (Morgan and Curran 1986). In nerve growth factor (NGF) differentiated PC12 cells, K<sup>+</sup>-induced depolarization (Morgan and Curran 1986) or treatment with the cholinergic agonist nicotine (Greenberg et al. 1985; Milbrandt 1986) results in a rapid increase in c-fos transcription. Depolarization-induced c-fos expression required the presence of extracellular Ca<sup>2+</sup> (Morgan and Curran 1986). This effect was mimicked by voltage dependent Ca<sup>2+</sup> channel agonists and was blocked by antagonists of this channel (Morgan and Curran 1986). These findings suggest that the voltage-gated increase in intracellular Ca<sup>2+</sup> may regulate c-fos expression in excitable cells.

Numerous studies have also examined c-fos activation in the mammalian brain. Morgan et al. (1987) have demonstrated that c-fos mRNA was transcribed after pentylenetetrazol (PTZ)-induced seizures in mice. Similar studies have shown an increase in c-fos

immunostaining in the nuclei of dentate gyrus granule cells after PTZ-induced seizures (Dragunow and Robertson 1987a) or electrical stimulation (Dragunow and Robertson 1987b; Sagar et al. 1988). Physiological (cutaneous) stimulation of rat primary sensory afferents results in c-fos immunoreactivity in the dorsal horn neurons of the spinal cord (Hunt et al. 1987). C-fos is also induced in the hypothalamic paraventricular and supraoptic nuclei, areas involved in thirst control, following 24 hr of water deprivation (Sagar et al. 1988). Further studies in the hypothalamus have demonstrated that light exposure can induce c-fos immunostaining in the suprachiasmatic nucleus (SCN) in rat (Rea 1989) and hamster (Rusak and Robertson 1989). All of these studies indicate that c-fos expression occurs after synaptic stimulation. This has led to the suggestion that c-fos immunostaining could be used as an anatomical marker of metabolic activity (Sagar et al. 1988; Dragunow and Faulk 1989) in a similar manner to 2-deoxyglucose (Schwartz et al. 1979). Such studies by Sagar et al. (1988) have demonstrated that electrical stimulation of the hindlimb motor/sensory cortex in the rat induces c-fos expression in brain areas known to be targets of motor/sensory output. This is a significant observation and indicates that c-fos activation is not necessarily confined to the first synaptic relay (Hunt et al. 1987), but can occur at least two synapses from the motor sensory cortex.

Although c-fos gene expression is induced by a wide range of stimuli in many cell types (Curran 1988), it is difficult to ascribe a specific role to these proteins because of the limited studies which have attempted to identify their target genes. Sonnenberg et al.

(1989a) have provided evidence, from studies in the hippocampus, that the proenkephalin gene may be regulated by Fos and Jun proteins. They demonstrated that Fos and Jun bind to the 5' AP-1-like regulatory region of the proenkephalin gene and that c-fos and c-jun cooperatively stimulate transcription from this region.

Morgan et al. (1987) originally reported that the pattern of PTZ-induced c-fos immunostaining was similar to the density of receptors for NMDA. This observation and evidence that NMDA receptors could gate  $Ca^{2+}$  (Ashcer and Nowak 1987) strongly suggested a role for the NMDA receptor in the activation of c-fos. Sonnenberg et al. (1989b) have examined the role of glutamate and c-fos expression and have shown that NMDA and kainate induce c-fos mRNA in mouse brain. Recently, Szekely et al. (1989) demonstrated that NMDA induced c-fos mRNA in primary cultures of cerebellar granule cells. Activation was prevented by the competitive NMDA antagonist APV and the non-competitive antagonists  $Mg^{2+}$  and PCP. Contrary to the report of Sonnenberg et al. (1989b), neither kainate nor quisqualate increased basal expression of c-fos mRNA. Similar experiments by Didier et al. (1989) and by Balazs et al. (1988a,b) have implicated a role for c-fos in the neurotrophic effects of NMDA on cerebellar granule cells in culture. Further evidence supporting a role for the NMDA receptor in the activation of c-fos is provided by Dragunow et al. (1990). This group observed that preinjection of the NMDA non-competitive antagonist MK-801 inhibited c-fos protein accumulation after traumatic brain injury.

Glutamate and the NMDA receptor have also been implicated as important regulatory components of the hypothalamic control of

luteinizing hormone (LH) secretion in the rat (Olney and Price 1980; Schainker and Cicero 1980; Urbanski and Ojeda 1987) and monkey (Gay and Plant 1987; Wilson and Knobil 1982; see also Sect. I Part D.-2). A critical issue in all of these studies relates to the precise location of the NMDA-specific glutamate receptors which regulate GnRH secretion. Experiments *in vitro* (Bourguignon et al. 1989) indicate that GnRH can be released from neuron terminals contained within the mediobasal hypothalamus (MBH). In contrast, others have suggested that NMDA has no effect *in vitro* (Donoso et al. 1990) or *in vivo* (Ondo et al. 1988) on the secretion of GnRH from the MBH. Indeed, Ondo et al. (1988) report that the medial preoptic nucleus (MPO) is the site from which endogenous glutamate controls GnRH secretion. Nevertheless, peripheral injection of prepubertal rats with NMDA rapidly induces maximal secretion of LH within eight minutes (Cicero et al. 1988a; see also Sect. I Results p. 81).

Experiments are presented here, in which cellular activity, in response to peripherally-injected NMDA, was immunocytochemically localized to individual cell nuclei via expression of *c-fos*. Because the induction of the *c-fos* gene is recognized as a sensitive marker of neuronal activity (Robertson and Dragunow 1990; Sagar et al. 1988; Sheng and Greenberg 1990) *c-fos* immunostaining may be particularly useful in the analysis of the GnRH neuronal system (Hoffman et al. 1990).

## Material and Methods

### *Animals*

Female Sprague-Dawley rats (Canadian Hybrid Farms, Halls Harbour, Nova Scotia, Canada) were obtained at age 21 days (day of birth designated as day 0), housed in plastic cages and maintained under a light:dark photoperiod of 14:10 hours, (lights on from 0700 hr - 2100 hr). For some experiments male rats were obtained at age 21 days or 60 days and housed 4-6 rats per cage. The room temperature was maintained at  $21\pm 1^{\circ}\text{C}$ . Unless otherwise noted, all animals received food (Purina Rat Chow) and drinking water *ad libitum*.

### *Drugs*

Monosodium glutamate (MSG) was generously provided by Dr. D. Nance (Dept. of Anatomy, Dalhousie University). N-methyl-D-aspartate (NMDA) was obtained commercially from Sigma Chemical Company, St. Louis MO. MK-801 and 2-amino-5-phosphonovalerate (APV) were purchased from Research Biochemicals Inc., Natick, MA. Sodium pentobarbital (Somnotol) was obtained from MTC Pharmaceuticals (Cambridge, Ontario).

### *Immunocytochemistry*

Two hours after drug treatment, rats were deeply anaesthetized with Somnotol (0.4 ml; i.p.) and perfused by inserting a cannula into the left ventricle of the heart. Phosphate buffered saline (PBS; 100 ml;  $4^{\circ}\text{C}$ ) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; 100 ml; pH 7.4) at room temperature were perfused into the rat using a peristaltic pump. Once perfused, brains were carefully

removed, after cutting the optic nerve, and were stored in 4% paraformaldehyde in PB at 4°C for at least 72 hr before sectioning. The brains were blocked by a single coronal cut approximately 3 mm rostral to the optic chiasm. Coronal sections (50  $\mu$ M) were then cut on a Vibratome with the brain immersed in PBS (4°C) during sectioning. Sections were placed into 2.5 ml mesh-bottomed sample cups (Fisher) that fit into individual, bottom-removed wells of a 24-well tissue culture plate (Falcon). The tissue culture plates holding the sample cups were then placed onto intact culture plates which contained the various solutions needed for immunocytochemistry. This method alleviated the problem of removing and replacing solutions from the same well containing the tissue sections i.e. the mesh-bottomed cups were simply removed from the solutions.

The sections were washed (10 min per wash) with PBS containing 0.2% triton x-100, followed by another wash in 0.3% H<sub>2</sub>O<sub>2</sub>, (this step removes peroxidase activity and blood within the cell) and then washed twice in PBS. Sections were subsequently incubated in 10% normal rabbit serum (Dimension Labs) for 20 mins to provide a block against any non-specific binding by the primary antibody. Sections were next incubated for 48 hr with the primary c-fos antibody at a dilution of 1/2000 (Cambridge Research Biochemicals, Cambridge, U.K.) in 1% rabbit serum made up in 0.01 M phosphate buffer containing 1% triton x-100 at 4°C. Sections were again washed 3 times with PBS containing 0.2% triton x-100 (10 min per wash) and incubated with biotinylated rabbit anti-sheep antibody (Vector Labs) for 1 hr, using a modification of the avidin-biotin technique (Dragunow and Robertson 1988). After washing sections 3 times in

PBS, they were incubated with the ABC solution (avidin-biotin peroxidase complex) (Vector) for 1 hr, rinsed 3 times in PBS and placed in the chromagen diaminobenzidine (DAB; Sigma) for 5 min. The peroxidase reaction was visualized with the addition of glucose oxidase to the medium for 45 min. This was accomplished by glucose oxidase reacting with the glucose within the DAB solution and producing  $H_2O_2$ . The resulting  $H_2O_2$  reacted with the peroxidase bound to biotin causing the DAB to change from colorless to red. This was followed by 3 more washes in PBS (10 min per wash). All incubations were performed at room temperature. Sections were mounted on subbed glass slides and allowed to dry overnight. Once sections were dry, they were dehydrated to 100% ethanol, cleared in xylene, and coverslipped.

## Experiments

### *1. The Effect of NMDA and MK-801 on the Induction of c-fos.*

Female rats (postnatal day 30) were injected with (a) the glutamate agonist, NMDA (s.c.; 15 mg/kg) (b) physiological saline (c) the NMDA non-competitive antagonist, MK-801 (0.1 mg/kg) followed 60 min later by NMDA (as in (a)) (d) MK-801 alone.

Two hours after NMDA/saline (3 hr post MK-801 injection) the rats were deeply anaesthetized, perfused and the brains removed for sectioning.

*2. The Effect of Neonatal Monosodium Glutamate (MSG)-induced Arcuate Lesions on the Induction of c-fos*

Newborn female pups (8 per litter) were injected with saline (0.1 ml; s.c.) or MSG (4 mg/g body weight) on postnatal days 2, 4, 6, and 8 or on days 2 and 4 only. On postnatal day 36, three rats from each litter were injected with saline (0.1 ml; s.c.) or NMDA (15 mg/kg; s.c.). These rats were perfused as described above, 2 hr post-injection.

*3. The Effect of NMDA and APV on the Induction of c-fos*

Female rats (postnatal day 30) were injected with (a) NMDA (15 mg/kg) (b) the competitive NMDA antagonist APV (10 mg/kg; s.c.), followed 30 min later by an injection of NMDA (c) APV (20 mg/kg) followed 30 min later by NMDA (d) APV (20 mg/kg) and NMDA simultaneously (e) APV alone. Two hours after NMDA injection (or APV for (e)) the rats were deeply anaesthetized, perfused and the brains removed for sectioning.

*4. The Effect of NMDA on the Induction of c-fos in Prepubertal and Adult Male Rats*

Male rats aged 25 or 60 days were injected with saline (0.1 ml; s.c.) or NMDA (15 mg/kg; s.c.). These rats were perfused 2 hr post-injection as described above.

## Results

### 1. Effect of NMDA on the Induction of c-fos Immunoreactivity in the Immature Female Rat

Figure 30A illustrates the pronounced NMDA-induced immunostaining for c-fos-like protein in cells of the arcuate nucleus and the median eminence. The result of saline injection is shown in Fig. 30B. The stimulatory effect of NMDA on c-fos-like immunoreactivity was completely prevented by pre-injection of MK-801 (60 min; 0.1 mg/kg) (Fig. 30C). The influence of MK-801 alone is shown in Fig 30D. The block of NMDA-induced c-fos-like immunoreactivity by MK-801 clearly implicates the NMDA receptor-coupled ion channel in the induction of the c-fos antigen (Monaghan et al. 1989).

### 2. Effect of NMDA on the Induction of c-fos Immunoreactivity in the Immature Female Rat Treated Neonatally with MSG

In an attempt to eliminate glutamate/NMDA receptors, newborn female rat pups were treated with MSG. The lower dose of MSG (4 mg/g; postnatal days 2 and 4) clearly attenuates the NMDA-induced increase in immunostaining (Fig. 30E). Note that the c-fos-like immunoreactivity in the median eminence has been markedly reduced. The higher dose of MSG (4 mg/g; postnatal days 2, 4, 6, and 8) produced a further reduction in c-fos immunostaining (Fig. 30F) leaving just a residual pattern at the ventrolateral edge of the arcuate nucleus.

**Fig. 30** Photomicrographs of the arcuate nucleus-median eminence region of the prepubertal female rat hypothalamus illustrating the location of c-fos protein-like immunoreactivity (scale bar = 200 $\mu$ M). (a) immunoreactive staining 120 min following an injection of NMDA (15 mg/kg; s.c.). (b) as in (a) but 120 min after physiological saline (0.1 ml; s.c.) (c) effect of MK-801 (0.1 mg/kg; s.c.) injected 60 min prior to an injection of NMDA. (d) MK-801 (0.1 mg/kg; s.c.) alone. (e) NMDA-induced immunoreactive staining in rats neonatally treated with MSG (4 mg/g; injected on postnatal days 2 and 4). (f) NMDA-induced immunoreactive staining in rats neonatally treated with MSG (4 mg/g; injected on postnatal days 2, 4, 6, and 8)

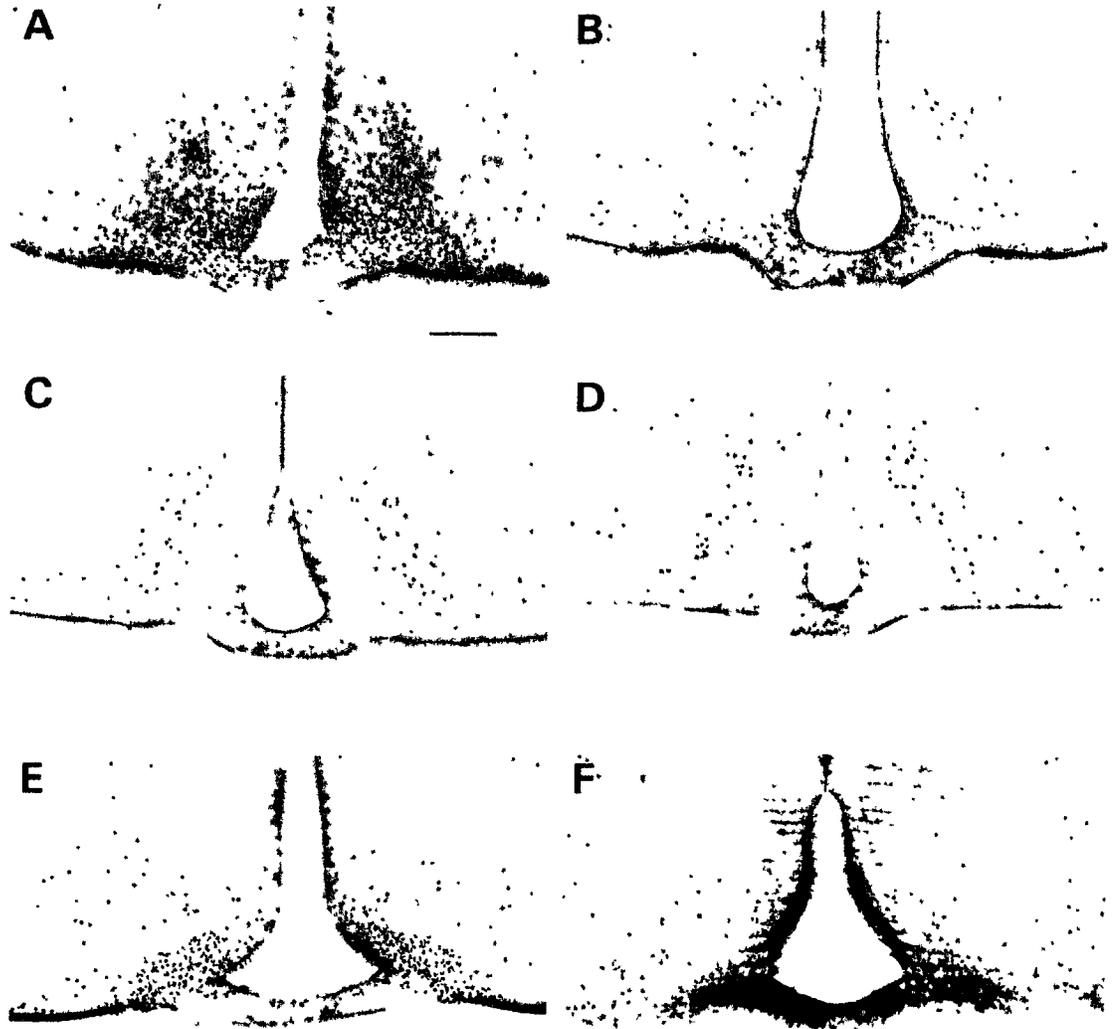


Figure 30

### 3. Effect of APV on NMDA-induced c-fos Immunoreactivity

When the NMDA-competitive antagonist APV (10 mg/kg) was injected 30 min pre-NMDA injection (15 mg/kg) almost all immunostaining in the MBH was eliminated (Fig. 31B) when compared to an NMDA-treated rat (Fig. 31A). However, when the dose of APV was increased to 20 mg/kg and pre-injected 30 min before NMDA treatment (Fig. 31C) or co-injected with NMDA (not shown), c-fos immunostaining in the arcuate and median eminence was completely eliminated. Fig. 31D demonstrates the effect of APV injection alone.

### 4. Effect of NMDA on the Induction of c-fos Immunoreactivity in the Subfornical Organ (SFO)

The localization of immunoreactivity to the arcuate-median eminence region is consistent with the likelihood that NMDA does not cross the blood-brain barrier (BBB) (Gay and Plant 1987). The subfornical organ, a circumventricular organ, like the MBH, is not protected by the BBB. Fig. 31E demonstrates the NMDA-induced increase in c-fos-like immunoreactivity in the subfornical organ. Pre-injection with MK-801 (described above) or co-injection with APV eliminated c-fos immunostaining in this area (not shown) and was identical to the effect of saline injection shown in Fig. 31F.

### 5. Effect of NMDA on the Induction of c-fos Immunoreactivity in the Immature and Mature Male Rat

The differences in NMDA-induced c-fos-like immunoreactivity in prepubertal (postnatal day 25) and adult (postnatal day 60) male

**Fig. 31** Photomicrographs of the arcuate nucleus-median eminence region of the immature female and male rat hypothalamus illustrating the location of c-fos protein-like immunoreactivity (scale bar = 200 $\mu$ M). (a) immunoreactive staining 120 min following an injection of NMDA (15 mg/kg; s.c.). (b) effect of APV (10 mg/kg; s.c.) injected 30 min prior to an injection of NMDA (15 mg/kg; s.c.). (c) effect of APV (20 mg/kg; s.c.) injected 30 min prior to an injection of NMDA (d) APV (20 mg/kg; s.c.) alone. (e) immunoreactive staining in the subfornical organ (SFO) 120 min following an injection of NMDA (15 mg/kg; s.c.). (f) as in (e) but 120 min after physiological saline (g) immunoreactive staining 120 min following an injection of NMDA (15 mg/kg; s.c.) in male rats on postnatal day 25. (h) immunoreactive staining 120 min following an injection of NMDA (15 mg/kg; s.c.) in male rats on postnatal day 63.

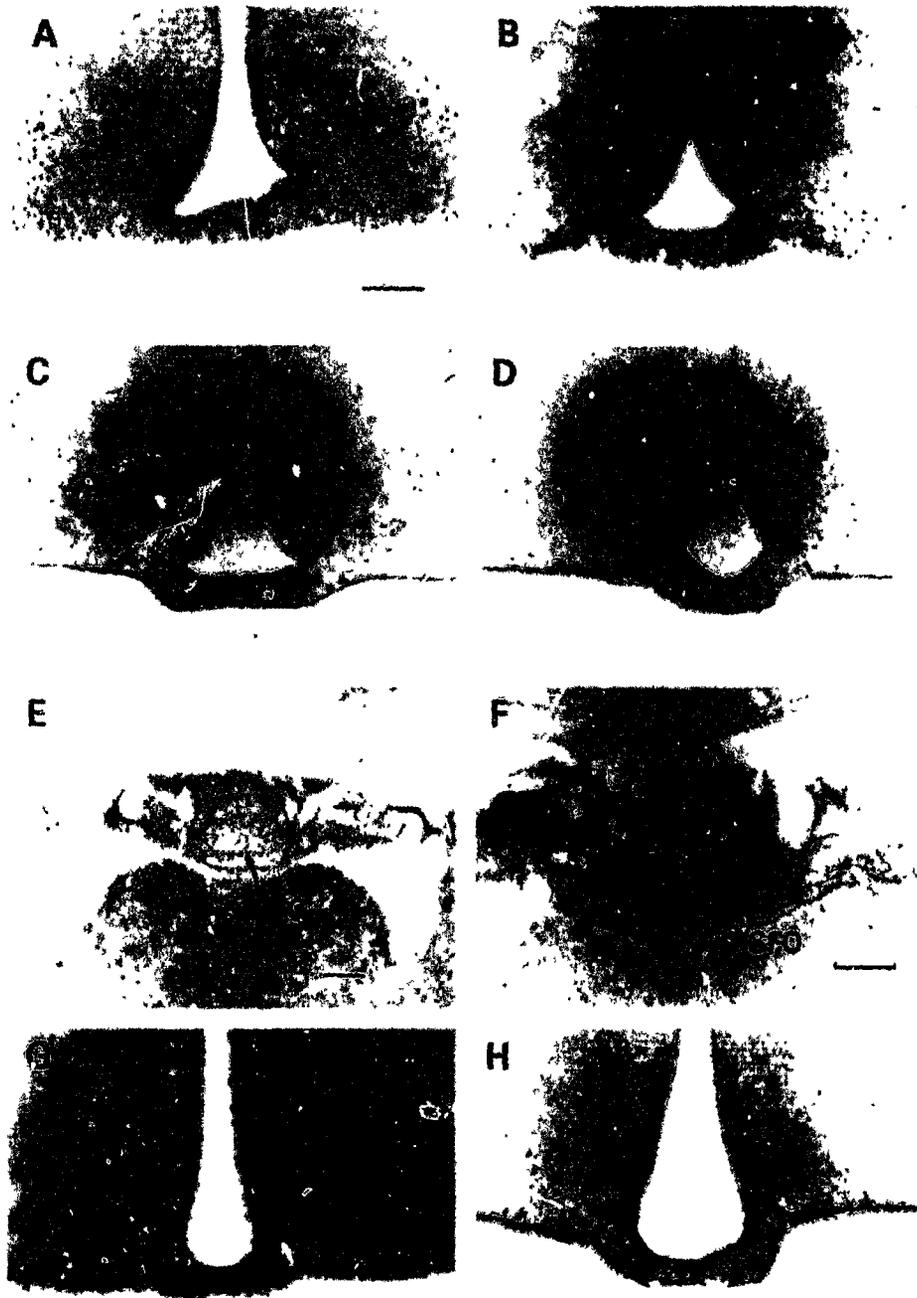


Figure 31

rats are shown in Fig 31G and 31H respectively. The effect of saline injection on c-fos immunostaining in the prepubertal male and adult control (not shown) was identical to that observed in the saline-injected prepubertal female rat (Fig. 30B). The NMDA-induced c-fos-like immunoreactivity in the young male was similar to that in the day 30 female, both demonstrating immunostaining throughout the arcuate-median eminence area. In contrast, NMDA-induced c-fos immunoreactivity in the older male was greatly reduced when compared to the younger male. C-fos immunostaining was sparsely scattered in the arcuate nucleus and almost non-existent in the median eminence.

## Discussion

These results demonstrate for the first time that NMDA, in addition to inducing LH secretion, also activates the c-fos proto-oncogene in an area of the brain known to be important for the regulation of GnRH release. However, at present, firm conclusions cannot be made as to the neurochemical identity of the neurons which express the c-fos gene in response to NMDA. Although the c-fos immunoreactivity in the MBH and SFO, two areas thought to mediate GnRH and subsequent LH secretion (Bourguignon et al. 1989; Donevan et al. 1989), implies that c-fos expression may have a role in the control of GnRH release, the actual relationship between c-fos gene activation and NMDA-induced LH secretion remains to be determined. Nevertheless it has been established here (see Sect. I Results p. 81) that LH release in prepubertal female rats is maximal at approximately 7 min following NMDA injection, and an increase in NMDA-stimulated c-fos mRNA is certainly detectable in less than 15 mins (Sonnenberg et al. 1989b; Szekely et al. 1989). Also in terms of specificity, the block of NMDA-induced c-fos immunostaining in the MBH and SFO by the NMDA antagonists APV and MK-801 suggests that activation of the NMDA receptor and its associated voltage dependent cation channel are responsible for c-fos activation in these areas. This parallels the requirements for LH release. The correlation between NMDA-induced LH secretion and c-fos staining suggest that c-fos immunoreactivity could be used to localize neurons that mediate the proestrous LH surge and first ovulation (Lee et al. 1990). Experiments supporting the hypothesis that c-fos immunoreactivity

can be induced by physiological stimuli have recently been reported (Rusak and Robertson 1989; Rea 1990). Both of these groups demonstrated that 15-30 min of bright light induces c-fos immunoreactivity in the suprachiasmatic nucleus of hamsters and rats, an effect that is blocked by pre-injection with MK-801.

MSG treatment kills a variety of neuronal cell types (Meister et al. 1989), any number of which may be c-fos positive. The pattern of c-fos immunoreactivity in rats treated with 4 mg/g on days 2 and 4 is not unlike the pattern of cresyl violet staining observed by Seress (1982). Both of these staining patterns, in rats neonatally treated with the same dose of MSG, demonstrate a "flattening" of the anterior arcuate nucleus towards the ventral surface of the MBH and a movement of the ventromedial nucleus from a dorso-lateral position to a more ventral position (Walaas and Fonnum 1978). Rats treated with the high dose of MSG demonstrated minimal c-fos staining in the arcuate and ME. This suggests that the excitotoxic effects of MSG, at least in terms of c-fos expression, are greater at a larger dose. It is therefore interesting that the lower dose of MSG used was more effective at inducing precocious puberty in these rats (see Sect. I Results p.66). Even more interesting is the observation that NMDA-induced LH secretion is the same in MSG treated rats as in saline injected controls (see Sect. I Results p. 92). One possible explanation for this result is that neurons which mediate GnRH and subsequent LH release may reside in a discrete area of the arcuate nucleus that is unaffected by MSG treatment (Seress 1982; Meister et al. 1989). Serial sections through the preoptic area and hypothalamus from

MSG-treated and control rats would be useful in locating such an area.

The outcome of the MSG experiments suggests that the c-fos positive neurons probably possess glutamate receptors which render these cells vulnerable to the excitotoxic effects of MSG. Experiments reported here (see Sect. I Results p. 81) have demonstrated that the neurotoxic influence of MSG can be prevented by treatment with MK-801. This provides further evidence that NMDA acts directly on the cells which express the c-fos proto-oncogene. Some arcuate neurons contain mRNA for the estrogen receptor (Simerly et al. 1990) which also seem to be sensitive to MSG (Jennes et al. 1984). Recent work (Cattaneo and Maggi 1990; Insel 1990) has suggested a relationship between the control of c-fos gene transcription and the presence of estrogen receptors. Such a correlation - between NMDA induction of c-fos and estrogen sensitive neurons- appears reasonable in view of the prominent role that estrogen fulfills in the glutamate-regulation of LH secretion (Lopez et al. 1990; Reyes and Ferin 1989; Urbanski and Ojeda 1990).

The decrease in NMDA-induced c-fos immunostaining in adult males compared to prepubertal males suggests a loss of NMDA receptors within the MBH (Cicero et al. 1988a; Tremblay et al. 1989). Experiments demonstrating an increase in the endogenous glycine antagonist kynurenic acid (Moroni et al. 1988) during development suggest that the decrease in c-fos immunoreactivity seen in older animals may result from decreased NMDA receptor binding and activation. However, an age-related decrease in NMDA receptor number (Tremblay et al. 1990) must also be considered. Reported

here (see Sect. I Results p. 95), and by others (Cicero et al. 1988a), are the age-related differences in the NMDA-induced release of LH. The observation that NMDA-induced LH release is much greater in prepubertal males than in adult males correlates well with the amount of c-fos staining seen at each age. Note, however, that female rats treated with high doses of MSG released significantly higher levels of LH after NMDA injection even though c-fos staining was almost non-existent. It is obvious that further experiments on NMDA-induced LH levels and c-fos immunocytochemistry are needed in the adult female rat if more valid comparisons are to be made.

In conclusion, the data presented here confirm the original suggestion (Sagar et al. 1988) that the immunohistochemical localization of c-fos gene expression promises to be a valuable technique for the mapping of glutamate sensitive pathways. In combination with the immunocytochemical localization of GnRH and estrogen-binding neurons (Hoffman et al. 1990), this technique could provide anatomical and physiological evidence that better defines the neural control of sexual maturation.

### Section III. OPIOIDERGIC CONTROL OF SEXUAL MATURATION

#### Introduction

The neural control of puberty is the subject of sustained investigation in several species, including the rat (Ojeda and Urbanski, 1988), sheep (Foster, 1988), and primate (Plant, 1988). Convincing evidence that glutamate may be an important excitatory influence that mediates the onset of puberty has been presented (see Sect. I Results and Discussion p. 52-110). This section will examine some of the evidence implicating the hypothalamic endogenous opioid peptide (EOP) system as one of the presumptive components of this maturational process (Wilkinson and Landymore, 1989; Cicero et al., 1986).

The Gonadostat theory (Ramirez and McCann 1963), discussed earlier (Sect. I Part A. p. 1) has been further redefined and expanded as a broader understanding of hypothalamic control of gonadotropin release emerged. For example, a large body of evidence suggests that, in the mature reproductive system, an opioid system exerts a tonic inhibitory control on the hypothalamic-pituitary-axis (Ferin et al. 1984; Bicknell 1985; Millan and Herz 1985; Thind and Goldsmith 1988). Therefore the excitatory drive responsible for pulsatile GnRH release in prepubertal animals may be kept in check by a central inhibitory opioid mechanism or brake (Reiter and Grumbach 1982; Ojeda et al. 1983; Sirinathsinghji et al. 1985; Wilkinson and Landymore 1989; Meijs-Roelof and Kramer 1989). The decrease in sensitivity to opioids (i.e. removal of the opioid brake), takes place in

parallel with the decrease in steroidal sensitivity as sexual maturation proceeds and, as proposed by Bhanot and Wilkinson (1983), may represent the specific neurochemical mechanism for the resetting of the gonadostat (opiostat?).

#### A. Steroids and EOP

Outlined in this way, an opiostat hypothesis must be dependent upon some type of interaction between sex steroid feedback and central EOP. Studies in the ovariectomized (OVX) lamb (Foster 1988) and female rat (Blank et al. 1979; Cicero et al. 1986) have demonstrated the disappearance of opioidergic control of LH secretion at puberty. The suggestion that sex steroids modulate the inhibitory control of EOP (Cicero et al. 1979; Bhanot and Wilkinson 1983; Petraglia et al. 1984) implies that the opioid system may represent a bridge between the negative feedback effects of gonadal steroids and GnRH release from the hypothalamus (Cicero et al. 1980). The concept that estrogen can lead to the decrease in  $\beta$ -endorphin ( $\beta$ -END) inhibition during sexual maturation may have anatomical and physiological correlates. Studies by Jirikowski et al. (1986) and Morrell et al. (1985) have shown that  $\beta$ -END immunoreactive neurons in the rat arcuate nucleus have the ability to concentrate  $E_2$  whereas the studies of Shivers et al. (1983) demonstrate that GnRH neurons (located in the preoptic area) do not possess estrogen receptors.

Experiments by Wardlaw et al. (1982a) have shown that sex steroids stimulate the release of  $\beta$ -END into the hypophyseal portal

blood and decrease the concentration of hypothalamic  $\beta$ -END (Wardlaw et al. 1982b; 1985). In agreement with these observations, ovariectomy without  $E_2$  replacement decreases  $\beta$ -END secretion (Ferin et al. 1984). Conversely, Sarkar and Yen (1985) have shown that during the LH surge, when  $E_2$  levels decrease,  $\beta$ -END concentrations in the hypophyseal portal blood decrease dramatically. This suggests that hypophyseal plasma concentrations of  $\beta$ -END represent an indirect measure of opioid tone within the hypothalamus. These studies suggest that differences in  $E_2$  concentrations result in a variable opioid tone within the hypothalamus. Precise reasons for the differences in  $\beta$ -END levels observed in the portal blood are unclear although methodological procedures should be taken into consideration (Herbert 1989). In any event,  $\beta$ -END levels in hypothalamus and in portal blood should reflect a combination of biosynthesis, degradation and secretion. Recent studies using *in situ* hybridization (Wilcox and Roberts 1985; Schacter et al. 1986; Tong et al. 1990), examined the effect of OVX and OVX with  $E_2$  replacement on POMC mRNA levels. Results indicate that  $E_2$  decreases POMC mRNA in the arcuate nucleus. Similar results using testosterone replacement in castrated males have also been observed (Blum et al. 1989). These studies suggest that the presence of sex steroids decreases  $\beta$ -END synthesis although release was not measured in these experiments. The decrease in POMC mRNA synthesis and decrease in hypothalamic  $\beta$ -END as a result of an increase in steroid hormones correlates well with a decrease in opioid tone required for the generation of the LH surge (Lustig et al. 1989; Masotto et al. 1990). However, the suggestion that  $E_2$  causes an

increase in  $\beta$ -END release (Wardlaw et al. 1982; Ferin et al. 1984) infers that a greater inhibitory tone as opposed to a reduction in opioid inhibition is actually present within the hypothalamus. Experiments designed to examine the levels of POMC synthesis combined with hypothalamic  $\beta$ -END content and release are certainly warranted. Those studies which investigate the post-translational processing of POMC and  $\beta$ -END are also relevant. Work by Martensz (1985), specifically in prepubertal rats, has shown that as female rats become sexually mature,  $\beta$ -END 1-31 (parent peptide) is proteolytically cleaved to two smaller fragments (1-26 and 1-27) that are without opioid activity (i.e.  $\beta$ -END content is decreased). The report that  $\beta$ -END (1-27) is a potent antagonist of  $\beta$ -END (Nicolas and Li 1985; Suh et al. 1988) and that  $\beta$ -END is inactivated as sexual maturation proceeds (Martensz 1985) could account for the puberty-related reduction of opioid peptide inhibition of LH secretion (Bhanot and Wilkinson 1983). A critical experiment would be to determine whether gonadal steroids might influence the processing of POMC to the smaller  $\beta$ -END fragments. It is clear that sex steroids represent a specific regulatory component of EOP during sexual maturation. The precise nature of this regulation is still undefined although much has been learned from the pharmacological manipulation of LH secretion with opiate agonists and antagonists in different steroid environments.

## B. EOP Antagonists

Advances in our understanding of opioidergic regulation of gonadotrophin releasing-hormone (GnRH) secretion owes much to the availability of the opiate antagonist drug naloxone (NAL). There appear to be profound age and sex differences in the LH response to NAL injections (Blank et al. 1979; Ieiri et al. 1979; Schulz et al. 1982; Bhanot and Wilkinson, 1983; Cicero et al. 1986; Almeida and Schulz, 1988; Becu'-Villalobos et al. 1989). In female rats NAL-induced LH release is detectable at five days after birth (Blank 1980) and is maximal at 10-15 days. LH levels then fall at 20 days, becoming indistinguishable from basal levels. Sensitivity to NAL is restored at day 25 (Cicero et al. 1986) and appears to be coincident with an increase in the availability of unbound, biologically active E<sub>2</sub> (Germain et al. 1987). The ability of NAL to increase serum LH levels declines after the day 25 peak and as sexual maturation proceeds. By day 60 (adult) the NAL-induced LH response is only 50% as large as the increase observed on days 10 and 25 (Cicero et al. 1986).

In males, conversely, NAL-induced LH secretion is not observed until after day 30 (Cicero et al. 1986), a time at which blood levels of testosterone begin to rise (Ramaley 1979). On the other hand, Bhanot and Wilkinson (1984) have shown that castration of male pups at postnatal day 24 reveals a NAL-induced elevation of LH secretion. This response is lost one week post-gonadectomy but can be induced again by steroid priming (Bhanot and Wilkinson 1984). These results suggest that the degree of EOP involvement in LH secretion is directly related to the level of steroid negative feedback.

Careful studies of adult animals using opiate antagonists have provided a greater understanding of the possible mechanisms responsible for the onset of puberty. Opiate antagonists are found to be effective in stimulating LH release in cycling or OVX-E<sub>2</sub> primed rats (Cicero et al. 1979; Piva et al. 1985; Sylvester et al. 1982). Observations that the NAL induced LH response is absent during the LH surge suggests that a decrease in EOP tone is essential for the initiation of the LH surge (Gabriel and Simpkins 1983; Gabriel et al. 1986; Piva et al. 1985). This may also be true at first ovulation. Opiate antagonist administration at proestrus (prior to the LH surge) will advance the time of the LH surge (Allen and Kalra 1986; Lustig et al. 1988a). The effect of the changing steroid milieu throughout the rat estrous cycle on the NAL-induced increase in LH levels has been demonstrated by Gabriel et al. (1983). Similar studies in humans and monkeys indicate the LH responsiveness to NAL is greatest when estrogen and progesterone levels are high (luteal phase) and lowest when estrogen levels are decreased (follicular phase) (Quigley and Yen 1980; Wehrenberg et al. 1982; Bernasconi et al. 1986). In OVX-E<sub>2</sub> primed rats, progesterone and NAL have quantitatively similar effects on the timing of the LH surge; i.e., when progesterone is administered on the day of the LH surge (high estrogen) it acts like an EOP antagonist to advance the timing of the LH surge (Barraclough et al. 1986; Lustig et al. 1988b; Masotto et al. 1990). Both NAL and progesterone are capable of modifying hypothalamic norepinephrine turnover (Wise et al. 1981; Adler and Crowley 1984) and both increase GnRH secretion from the perfused proestrous hypothalamus *in vitro* (Leadem et al. 1985; Kim and

Ramirez 1986). Lustig et al. (1988b) suggest that progesterone advances the timing of the LH surge via genomic actions on the EOP-containing neurons (i.e. reduces their tonic activity). However, when progesterone is administered on the day before proestrus (low estrogen levels) the LH surge is suppressed, an effect that may result from an increased opioid tone (Wilcox and Roberts 1985).

Discussed earlier (Sect. I Part A p. 3) was the observation that the onset of sexual maturation in the female rat is signalled by the development of an increase in the amplitude of afternoon LH pulses (Urbanski and Ojeda 1985). These afternoon pulses eventually become minisurges of LH which stimulate E<sub>2</sub> production and subsequently the proestrus LH surge (Urbanski and Ojeda 1986). These diurnal variations in gonadotropin secretion may coincide with diurnal changes in opioid receptor populations (Jacobson and Wilkinson 1986; Wirz-Justice 1987). Blank and Mann (1981) demonstrated NAL-induced LH secretion throughout the day except between 15:00 hr and 18:00 hr. Although this suggests that opioid tone is reduced in the late afternoon, permitting the release of LH and an eventual LH surge, a quotidian increase in the afternoon response of the pituitary to GnRH must also be taken into account (Wilkinson and Moger 1981). Jacobson and Wilkinson (1986) have confirmed the observations of Blank and Mann (1981) and have also demonstrated that the diurnal variation in NAL effectiveness appears between day 23 and day 26 after birth. These experiments also indicated that the minimal response to NAL is coincident with a similar change in opiate receptors in the mediobasal hypothalamus as shown by an afternoon decrease in [<sup>3</sup>H]NAL binding. The reduction

in hypothalamic [<sup>3</sup>H]NAL binding was due to a decrease in receptor density and not a result of diurnal variations in receptor occupancy.

### C. The Effect of EOP Antagonists on LH Secretion in Other Species

The opioidergic control of LH secretion during sexual maturation has been examined in a number of different species. These include: the lamb (Foster et al. 1986; Foster 1988); the rhesus monkey (Plant 1988; Medhamurthy et al. 1990); female pigs (Barb et al. 1986; Barb et al. 1989); bull calves (MacDonald et al. 1990); the cockerel (Stansfield and Cunningham 1988); and humans (Ulloa-Aguirre et al. 1988; Mauras et al. 1986; Bernasconi et al. 1986; Bourguignon 1988). This section will concentrate on studies examining the ewe lamb and the rhesus monkey.

The neuroendocrine events that mediate seasonal changes in fertility in the adult sheep are similar to those controlling puberty in the lamb. Thus, the seasonal breeder has been extensively used as a model for puberty (Foster 1988). The onset of sexual maturation in sheep results from an increase in the frequency of episodic LH secretion that occurs at approximately 30 weeks of age. Removal of the ovaries at 3 weeks of age results in high LH pulse frequency by 6 weeks of age (Ebling and Foster 1989a). Estrogen replacement at the time of ovariectomy eliminates the high LH secretion until 25 weeks of age when sensitivity to E<sub>2</sub> negative feedback decreases. These results suggest that during the prepubertal period ovarian steroids are required for the suppression of LH secretion. This group also

demonstrated that NAL injections (4 x 1 mg/kg; i.v.) resulted in an increase in pulsatile LH secretion. This effect, however, was not seen in the mature seasonally anestrous sheep. These results lead to the conclusion that opioid pathways are an important modulatory influence on LH secretion during sexual maturation but not in the mature seasonally anestrous sheep. Recently, this group (Ebling et al. 1989b) performed experiments on OVX-E<sub>2</sub> (12 weeks of age) replaced lambs. In these experiments E<sub>2</sub> implants were removed 3 weeks after OVX (15 weeks of age) and NAL treatment was initiated at 18 weeks (prepubertal). In the absence of E<sub>2</sub>, lambs still showed an increase in LH pulse frequency. Estradiol implants were returned but then removed again on week 35 (postpubertal) and NAL treatment initiated on week 38. Lambs, again, demonstrated an increase in LH pulse frequency. These results imply that, although EOP may be an important modulatory influence on LH secretion during sexual maturation, it is unlikely that they mediate the decrease in steroid negative feedback and increase in pulsatile gonadotropin secretion that occurs at puberty.

Although the majority of studies using the rhesus monkey suggest that EOP are important regulators of the menstrual cycle i.e. they inhibit LH secretion at the level of hypothalamus in a steroid dependent manner (Ferin et al. 1984; Ferin 1987), the role of EOP during sexual maturation in primates is less clear. In higher primates, the ontogeny of gonadotropin secretion is characterized by elevations during infancy and adulthood that are separated by a period, or hiatus, of diminished LH secretion (Teresawa et al. 1983; Plant 1988). In the female rhesus monkey, the prepubertal hiatus

lasts for approximately 20 months and is followed by two major pubertal landmarks, menarche (30.5 months) and first ovulation (46.7 months). During the peripubertal stage (20-60 months) basal levels and pulse amplitude of LH increase. The development of a nocturnal increase in LH levels also occurs. Identical patterns of GnRH secretion have also been observed during this stage of development (Teresawa et al. 1989). Teresawa et al. (1984) demonstrated that neonatally OVX monkeys develop the same peripubertal LH pattern as intact animals, and suggest that the onset of puberty may be due to maturational changes of the hypothalamus (i.e. synaptogenesis) that are independent of ovarian steroid feedback. In an attempt to examine the role of EOP in the prepubertal hiatus of gonadotropin secretion in the rhesus monkey, Medhamurthy et al. (1989) examined the effect of NAL treatment on castrated prepubertal males. Although a single i.v. bolus or i.v. infusion of NAL increased LH in a number of the animals, this group still concluded that the prepubertal restraint of pulsatile GnRH release in primates, during the hiatus, is imposed by non-EOP pathways. The possible role of EOP in the peripubertal period of female monkeys has not been examined. Preliminary experiments by Blank et al. (1986b) have examined the LH response in male chimpanzees aged 1-9 years (puberty at 6-9 years). They demonstrated that only pubertal animals responded to NAL treatment with significant increases in LH. This NAL response is not unlike that in the male rat (Cicero et al. 1986). It seems to be dependent on a minimal amount of circulating testosterone which again emphasizes that the opioid control of LH release may be steroid

dependent. Interestingly, experiments by Thind and Goldsmith (1988), offer anatomical evidence in support of an EOP restraint on GnRH secretion. They demonstrated that POMC axons impinge directly on GnRH somata and proximal axons in the MBH of juvenile female monkeys. Note that this is the period of the prepubertal hiatus in gonadotropin secretion, a time when monkeys do not respond to NAL (Medhamurthy et al. 1989; Blank et al. 1986b).

In conclusion, it is still very much an open question whether EOP perform an important regulatory function in mammalian puberty. For a detailed critical survey of this field the reader is referred to Wilkinson and Landymore (1989).

#### **D. EOP Antagonists and Sexual Maturation**

If EOP do exert an inhibitory influence over GnRH release and therefore restrain the rate at which sexual maturation occurs, prepubertal treatment with opioid antagonists such as NAL or naltrexone should advance sexual maturation. Experiments performed by Sirinathsinghji et al. (1985) demonstrated a precocious maturation in female rats injected with naloxone every six hours during the first 10 days of life. However, other investigators have had difficulty replicating their results (Meijs-Roelofs and Kramer 1988; Landymore and Wilkinson 1988), although the latter authors indicated that a subgroup of rats did reach first ovulation precociously as a result of NAL treatment. The varied results between investigators are difficult to explain although the different

rat strains used by these groups may partially account for the diverse observations.

Although NAL effectively blocks hypothalamic opioid receptors, *ex vivo* binding experiments indicate that it only remains bound to the receptor for approximately 60 minutes (Landymore and Wilkinson 1988). Thus intermittent injections of NAL may not constitute a chronic blockade of opioid receptors as suggested by Sirinathsinghji et al. (1985). Naltrexone, an opioid antagonist reported to remain bound to hypothalamic [<sup>3</sup>H] -DAGO ( $\mu$ -opioid) receptors for as long as 15 hours (Landymore and Wilkinson 1988) has therefore been used for similar studies. Experiments by Lira et al. (1986) demonstrated that when naltrexone was injected into female rats from postnatal days 1-10 (the critical period in brain sexual differentiation) the development of E<sub>2</sub>-positive feedback action on LH release became impaired during the prepubertal period (postnatal day 24). Neonatal naltrexone treatment also delayed sexual maturation (Sarkar and Lira 1984) although it is unclear why the impairment of E<sub>2</sub> positive feedback did not completely prevent first ovulation. In more recent studies, when female rat pups were treated neonatally with naltrexone (Day 0-9; 50 mg/kg per day) no differences in the timing of V.O. were observed (Landymore and Wilkinson 1988; Meijs-Roelofs and Kramer 1988). However, when rats were injected between days 28 and 32 (20 mg/kg; 4 daily injections) 40% of the treated rats experienced advanced V.O. at significantly lower body weights (Meijs-Roelofs and Kramer 1988). More detailed studies examining the effects of naltrexone during the late juvenile period have been performed by this group (Meijs-Roelofs and Kramer

1989). They demonstrated that naltrexone (20 mg/kg; 4 daily injections) administered at postnatal days 30-34 advanced V.O. by 3.4 days in 75% of the treated rats. Body weights at first ovulation were also significantly reduced. When treatment was initiated before postnatal day 30 (i.e. day 26) or after (i.e. day 32) sexual maturation was not advanced. This group also demonstrated that the LH response to naltrexone was greatest on postnatal day 30 and suggest that during postnatal days 30-34 EOP critically restrict LH secretion and therefore may represent a hypothalamic restraint controlling the onset of puberty. In contrast, earlier studies using naloxone (Jacobson and Wilkinson 1986; Cicero et al. 1986) revealed the greatest increase in LH to occur at approximately day 26 while the lowest levels were observed at day 30. These differences are most likely rat strain-specific as the mean day of V.O. for control rats was also shifted (Meijs-Roloefs: 38 days; Wilkinson: 34 days). A thorough study of the possible differences in LH response to naloxone and naltrexone is also needed.

The use of opioid antagonists such as NAL and naltrexone have proved to be invaluable tools in implicating the EOP as neuromodulators of GnRH release and sexual maturation. Less attention has been given to the effect of opioid agonists on the hypothalamic-pituitary-axis, although morphine and the opioid peptide, FK 33-824 have been used to gain insight into the opioid control of LH secretion.

### E. Opiate Agonists and the Modulation of GnRH Secretion.

The majority of evidence indicates that the mu opioid receptor plays the most significant role in mediating the effects of opioids on GnRH secretion (Kalra and Leadem 1984; Cicero 1984; Bicknell 1987). Among the different EOP,  $\beta$ -END is thought to be the endogenous opioid that interacts at this receptor. Interestingly, both anti- $\beta$ -END (i.v.) (Forman et al. 1983) and anti-dynorphin (1-13) (kappa receptor ligand) (Schulz et al. 1981) - injected intracerebrally - increase LH secretion, with the former being somewhat more effective. Studies by Cicero et al. (1988b) using prepubertal (day 30) and adult (day 60) male rats, demonstrate that mu, kappa, and delta agonists are all capable of suppressing LH levels at both stages of development. Conversely, antagonists for each receptor were unable to induce LH release in young rats, whereas mu and kappa antagonists were able to increase LH in older rats. These authors suggest that the opiate antagonists either have no affinity for the opiate receptors in prepubertal males or, that the relevant EOP ligands are absent. Note however that the decreased steroid milieu in the younger rats may play an integral role in the inability of the various opiate antagonists to elicit an LH response (Bhanot and Wilkinson 1984).

Opiate agonists have also been used to provide greater insight into the ontogeny and location of the opioid control of LH release. Intracerebral injection of morphine ( $3\mu\text{g}/0.2\ \mu\text{l}$  of solvent) (Lakhman et al. 1989) into the medial preoptic area or the MBH on the day of proestrus, inhibited ovulation in cycling female rats. Rats

injected dorsomedially to the anterior commissure or lateral to the ventromedial hypothalamus, ovulated normally.

The effects of opiate agonists on LH secretion in the developing male and female rat are best exemplified by the work of Cicero et al. (1986). This study demonstrated that in male rats, the NAL-induced increase in LH is not seen until postnatal day 30, whereas morphine is able to inhibit LH secretion as early as day 15. Conversely, in the female rat, the ability of NAL to induce an increase in LH secretion occurs as early as day 10 but then decreases just prior to sexual maturation, whereas morphine does not decrease LH levels until the onset of puberty. Reasons for the differences in the LH response to NAL and morphine have not been well defined, although the possibility of different opioid receptors (i.e. kappa receptors) binding with opiate agonists or antagonists has been suggested (Schulz et al. 1981). Nevertheless, it appears that the maturation of the EOP-mediated control of GnRH and LH secretion occurs at different rates in the male and female rat and seems to be associated with the onset of sexual maturation (Cicero et al. 1986).

The role of the pituitary must also be taken into consideration. Although numerous reports have demonstrated that opiates and opioid peptides do not act at the anterior pituitary to decrease LH release (Millan and Herz 1986; Cicero et al. 1987; Bhanot and Wilkinson 1983), studies by Blank et al. (1986a) have shown that lengthy incubation with morphine is able to inhibit both basal and GnRH-stimulated LH release in cultured rat pituitary cells. This effect could be reversed by naltrexone or  $\beta$ -END anti-serum. Similar studies using human  $\beta$ -END to decrease LH release from pituitary cells *in*

*vitro* have also been reported (Sanchez-Franco and Cacicedo 1986). The effects of opiate (opioid) agonists and antagonists on GnRH secretion in perfused hypothalamic tissue should provide a clearer picture of the opioid regulation of GnRH secretion, especially during the onset of sexual maturation.

#### **F. Opiate Agonists and Sexual Maturation**

The knowledge that opiate agonists such as heroin can severely derange the mature reproductive system antedates the discovery of the EOP. However, the susceptibility of sexual maturation to opiate agonist inhibition is an aspect that has received little scrutiny. Cicero et al. (1989) have also drawn attention to this anomaly and have emphasized its importance in the context of drug abuse in the prepubescent and adolescent periods. Since morphine is also an endogenous opiate (Cardinale et al. 1987), of unknown function, further investigation of opiate agonist regulation of gonadotrophin secretion should provide important complementary data to those obtained from opiate antagonist studies. Some evidence already exists on the effects of morphine on sexual maturation in rats. Weiner and Scapagnini (1974) reported that morphine (2 mg/kg/day; from day 20) unexpectedly induced a small advancement in the time of vaginal opening (V.O.). A more prolonged drug schedule was employed by Zimmerman et al. (1974). Pups were injected with increasing doses of morphine from birth until weaning. The drug induced a slight but significant reduction in body weight but had no effect on the time of puberty in males or females.

Lintern-Moore et al. (1979) noted that large doses of morphine (50 mg/kg/day) given to 21-day-old female rats for 1 or 7 days had no effect on body weight but significantly reduced the initiation of follicular growth. It is reasonable to assume an involvement of the hypothalamus despite the fact that pituitary, ovarian and uterine weights were unaffected. This was confirmed by Advis et al. (1982). Immature female rats (postnatal day 23), implanted with osmotic minipumps that delivered morphine (1 $\mu$ g/hr), reached VO and first diestrus approximately 6 days later than controls. Continuous treatment thus seems to be more effective than intermittent daily injections in affecting the time of V.O. Implantation of morphine pellets close to the time of puberty exerted a marked inhibitory effect on the developing reproductive system of male rats (Cicero et al. 1989). In contrast, when single injections of morphine are given close to the expected time of the first LH surge a remarkable biphasic effect is observed (Hulse and Coleman 1983). First ovulation was induced by an injection of pregnant mare serum gonadotrophin (PMSG). Doses of 24 and 36 mg/kg of morphine, given on presumptive proestrus, inhibited ovulation rate (measured as number of ova) whereas 6 mg/kg significantly increased this rate. The low dose of morphine also increased the number of rats which ovulated after a sub-optimal PMSG stimulus. The most likely explanation of these data is that low doses of morphine are somehow able to increase LH secretion. This remains to be proven although opiates are reported to stimulate LH secretion under certain circumstances (Pang et al. 1977; Motta and Martini 1982; Cicero 1987; Van Vugt et al. 1989).

Previous work in this laboratory (Bhanot and Wilkinson 1983) demonstrated that an opioid agonist peptide, FK-33-824, becomes progressively less able to inhibit LH secretion as puberty approaches. These experiments suggested that a prepubertal decline in hypothalamic sensitivity to EOP may be a component of the neural mechanism which regulates the timing of first ovulation. Experiments with readily available drugs will be described in an attempt to further quantify this phenomenon. Prepubertal female rats were chronically exposed to opiate  $\mu$ -agonist drugs (fentanyl or morphine) via the drinking water or via osmotic pumps. The results are presented in this Thesis. (see Sect. III Results ).

## Materials and Methods

### *Animals*

Female Sprague-Dawley rats (Canadian Hybrid Farms, Halls Harbour, Nova Scotia, Canada) of specified ages (day of birth designated as day 0) were housed in plastic cages and maintained under a light:dark photoperiod of 14:10 hours, (lights on from 0700 h - 2100 h). The room temperature was maintained at  $21 \pm 1^\circ\text{C}$ . Unless otherwise noted, all animals received food (Purina Rat Chow) and drinking fluid *ad libitum*.

### *Drugs*

Morphine sulphate and fentanyl citrate were generously provided by BDH Chemicals, Toronto, Ontario and by McNeil Laboratories (Canada) Ltd., Stouffville, Ontario, respectively. The opiate antagonist naloxone hydrochloride was obtained from Sigma Chemical Company, St. Louis, MO., U.S.A.

### *Drug Administration*

#### Oral route

Fentanyl and morphine were administered at varying doses via the drinking water. Water was acidified to pH 2.5 with 5N HCl to prevent bacterial growth (Gordon et al. 1986) and sweetened with sucrose (50 g/L) to mask the bitter taste of the drug (Leung et al. 1986ab). Rats were gradually adapted to morphine by administering it in increasing concentrations (24 hrs apart) of 100, 200, 400, and finally 800 mg/L beginning on day 22. Animals were maintained on 100,

400, or 800 mg/L until the day of vaginal opening (V.O.) unless otherwise stated. In our colony, greater than 90% of rats ovulate coincident with V.O. The same step-up schedule was used to increase the concentration of fentanyl citrate. Beginning on day 22, fentanyl was administered in increasing concentrations (24 hrs apart) of 4.8, 9.6, 19.2, and 30 mg/L. Animals were maintained at each concentration until the day of VO unless otherwise stated. Control rats received acidified water sweetened with sucrose.

#### Subcutaneously Implanted Osmotic Pumps

Fentanyl citrate was administered using subcutaneously (sc) implanted osmotic minipumps (Alza Corporation, Palo Alto, California) a method of drug delivery that ensures a continuous rate of administration. Initially, pump model 2001 (delivery rate  $1\mu\text{l/hr}$  for 7 days) was implanted on day 24 and replaced with the same model on day 31. In subsequent experiments, drug administration was prolonged by implanting model 2002 (delivery rate  $0.5\mu\text{l/hr}$  for 14 days) on day 31. All pumps were implanted under light ether anesthesia and incisions were closed with stainless steel wound clips. Control rats were surgically prepared but did not receive pumps. Before osmotic minipumps were implanted, animals were partially habituated to the drug by receiving increasing concentrations of fentanyl in their drinking water. i.e. on day 22, animals were administered 4.8 mg/L of drug which was increased to 9.6 mg/L on day 23.

### *LH Radioimmunoassay*

The details of this assay can be found in Section I Materials and Methods, p. 39-43

### *Withdrawal Signs*

The development of physical dependence on morphine and fentanyl citrate was estimated by the naloxone precipitated withdrawal syndrome (Badawy et al. 1982). Naloxone HCl (2.5 mg/kg) dissolved in 0.1 ml of saline was injected sc into a total of 5 rats from each treatment group on the day of VO just prior to sacrifice. Immediately after the naloxone injection the following behavioral parameters were observed for 30 minutes: wet dog shakes, teeth chattering, chewing, writhing, diarrhea, ptosis, scream on touch, and hostility on handling.

## **Experiments**

### *1. Effect of Morphine on the Timing of V.O.*

Beginning on day 22, groups of rats (n=10) were administered different concentrations of morphine dissolved in the drinking water. Final concentrations were attained by day 25 (100, 400, or 800 mg/L). Body weights were recorded every 2 days until the day of vaginal opening (V.O.). Rats were either sacrificed, and reproductive organs removed and weighed, or were maintained on morphine for 10 days during which time wet vaginal smears were taken twice daily.

## 2. *Effect of Morphine on Adult Estrous Cyclicity*

Regularly cycling (2 consecutive 4 day cycles) adult female Sprague Dawley rats (50 days old; n=6) were given 200 mg/L for 2 days after which the concentration was increased to 400 mg/L and maintained for 11 days. After this period the morphine concentration was increased to 800 mg/L for 9 days. Vaginal smears were taken twice daily before and during morphine administration. Controls (n=6) received just sucrose in their drinking water.

## 3. *Effect of Fentanyl on the Timing of V.O.*

Beginning on day 22, groups of rats (n=10) were given different concentrations of fentanyl dissolved in the drinking water with final concentrations being attained by day 25 (4.8, 9.6, 19.2, 30 mg/L). Rats were weighed every 2 days and on the day of V.O., were sacrificed and the reproductive organs were removed and weighed. Some rats were maintained on fentanyl and had wet vaginal smears taken twice daily for 10 days.

## 4. *Effect of Fentanyl on Adult Estrous Cyclicity*

Regularly cycling (2 consecutive 4 day cycles) adult female Sprague-Dawley rats (n=6) were given increasing concentrations of fentanyl until final concentrations of 30 mg/L and 40 mg/L were attained. These concentrations were maintained for 15 days during which wet vaginal smears were taken twice daily. Body weights were recorded every 2-3 days. Controls (n=6) received just sucrose in their drinking water.

5. *Effect of Fentanyl via Osmotic Minipumps (2 x 7-day pump) on the Timing of V.O.*

This experiment investigated the effect of fentanyl citrate released from osmotic minipumps. On day 22 animals (n=10) received 4.8 mg/L of fentanyl citrate in their drinking water which was increased to 9.6 mg/L on Day 23. On the morning of Day 24 osmotic minipumps (Model 2001) containing fentanyl citrate (4.5 µg/hr) were surgically implanted as previously described (above) and drug was removed from the drinking water. On day 31 this pump was removed and replaced with a new pump (Model 2001), containing the same concentration of fentanyl (4.5 µg/hr). This concentration of fentanyl is equivalent to 1.8 mg/kg/day on the first day of implantation (day 24) and decreases as the animal becomes heavier. Body weights were recorded every 2 days until the day of VO. Rats were sacrificed and reproductive organs were removed and weighed. Controls (n=10) received an incision closed by a wound clip.

6. *Effect of Fentanyl via Osmotic Minipumps (14-day pump) on the Timing of V.O.*

This experiment is identical to the one above except on day 31 pump model 2002 (14 day pump) containing an increased concentration of fentanyl equivalent to 1.8 mg/kg/day (6.7 µg/hr) was implanted to compensate for the increased body weight of the animals. Again, body weight changes were monitored. On the day of VO, animals had wet vaginal smears taken twice daily.

*7. Effect of Fentanyl via Osmotic Minipumps (14-day pump) and MK-801 Injections on the Timing of V.O.*

This experiment investigated the effect of fentanyl citrate released from osmotic minipumps. On day 22 animals (n=10) received 4.8 mg/L of fentanyl citrate in their drinking water which was increased to 9.6 mg/L on Day 23. On the morning of Day 24 osmotic minipumps (Model 2002) containing fentanyl citrate (4.5 µg/hr) were surgically implanted as previously described (above) and drug was removed from the drinking water. Controls (n=10) received an incision closed by a wound clip. Beginning on postnatal day 27, animals were injected with the non-competitive glutamate antagonist MK-801 (0.1 mg/kg s.c. 1:00-2:00 pm) or saline until the day of V.O. On the day of V.O., animals were sacrificed and the ovaries removed and weighed.

*8. Effect of Fentanyl via Osmotic Minipumps (14-day pump) on Adult Estrous Cyclicity*

Adult female rats (n=5) exhibiting two consecutive 4 day cycles received increasing concentrations of fentanyl in the drinking water as described in the previous experiment. After 2 days, fentanyl was removed from the water and osmotic minipumps (model 2002), containing a concentration of fentanyl (15 µg/hr) equivalent to 1.8 mg/kg/day, were implanted as previously described. The day after implantation wet vaginal smears were taken twice daily for 14 days. Body weight changes were monitored every 3 days.

9. *Effect of Fentanyl via the Drinking Water on Basal LH Secretion prior to V.O.*

Beginning on day 22, groups of rats (n=10) were given fentanyl dissolved in the drinking water with final concentrations being attained by day 25 (30 mg/L). Controls received just sucrose in their drinking water. On postnatal days 25, 27, 29, 30, 32, and 34 between 9:00-11:00 am, groups of fentanyl treated (n=10) and control (n=9-10) rats were decapitated and trunk blood collected. Reproductive organs were removed and weighed on the day of sacrifice.

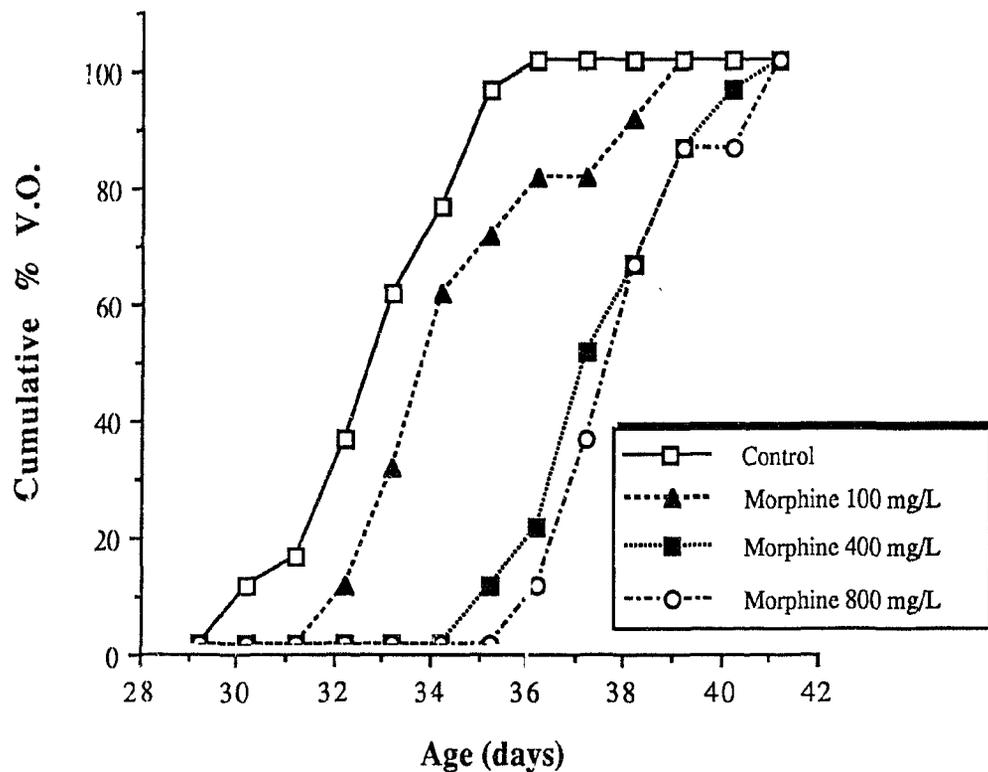
**Statistics**

Determination of significant differences between group means was accomplished using Student's *t* -test or analysis of variance (ANOVA) and a post hoc Dunnett *t*-test with  $p < 0.05$  denoting a significant difference.

## Results

### 1. Effect of Morphine on the Timing of V.O.

Based on a daily fluid (morphine + sucrose + water) intake of 250 ml/kg/day (data not shown) and an oral bioavailability of 20% (Dahlstrom and Lennart, 1978), animals administered 100, 400, and 800 mg/L of morphine consumed approximately 5, 20, and 40 mg/kg/day respectively. Figure 32 illustrates that V.O. is delayed by the two high doses of morphine sulphate when administered in the drinking water. Table 11 shows that this delay in the timing of V.O. was significant at higher concentrations of morphine (400 and 800 mg/L). Note that all three doses affected body weight at the time of puberty, whereas absolute uterine, ovarian and pituitary weights were unchanged by treatment. The growth curves (Fig. 33) indicate that morphine treatment resulted in an initial decrease in the growth rate, over a 2-day period, which then returned to normal. At the time of V.O. the body weights were significantly greater in all of the treated groups (Table 11). In two separate experiments, greater than 80% of the animals in each group ovulated with a normal complement of corpora lutea, while the remaining rats were in proestrus. In a further experiment, animals were not sacrificed but morphine treatment was continued. Vaginal smears were taken twice daily beginning immediately after V.O. Rats given 100 mg/L of morphine, before and after V.O., cycled normally (4 days/cycle). At concentrations of 400 and 800 mg/L of morphine, 83% (10/12) and 80% (8/10) of the rats respectively, remained in diestrus for a mean of 6 days after V.O. These rats then began to cycle regularly by 8



**Fig. 32.** Effect of different concentrations of morphine sulphate (100 mg/L; 400 mg/L; 800 mg/L; from day 22 until V.O.;  $n=10$ /treatment), dissolved in acidified sweetened drinking water. Data plotted as the cumulative % of rats showing V.O. at each age.

**Table 11.** The effect of morphine via the drinking water on the age at V.O. in immature female rats.

	n	Age (days)	Body Wt. (grams)	Uterus (mg/100 g B.W)	Ovaries (mg/100 g B.W.)	Ant. Pit. (mg/100 g B.W.)
Control	10	33.6±0.9	106.8±3.9	161.1±9.8	30.1±1.7	3.9±0.2
Morphine (100mg/L)	10	34.8±0.7	122.3±2.9***	145.7±14.1	27.8±1.8	3.2±0.5
Morphine (400mg/L)	10	38.2±0.5***	122.6±2.8***	140.2±11.3	27.6±1.6	3.3±0.4
Morphine (800mg/L)	10	38.5±0.4***	123.0±2.1***	130.7±21.0	27.1±2.6	3.2±0.2

Values are mean ±s.e.m.

\*\*\* p<0.01 when compared to controls; ANOVA and post hoc Dunnett t-test

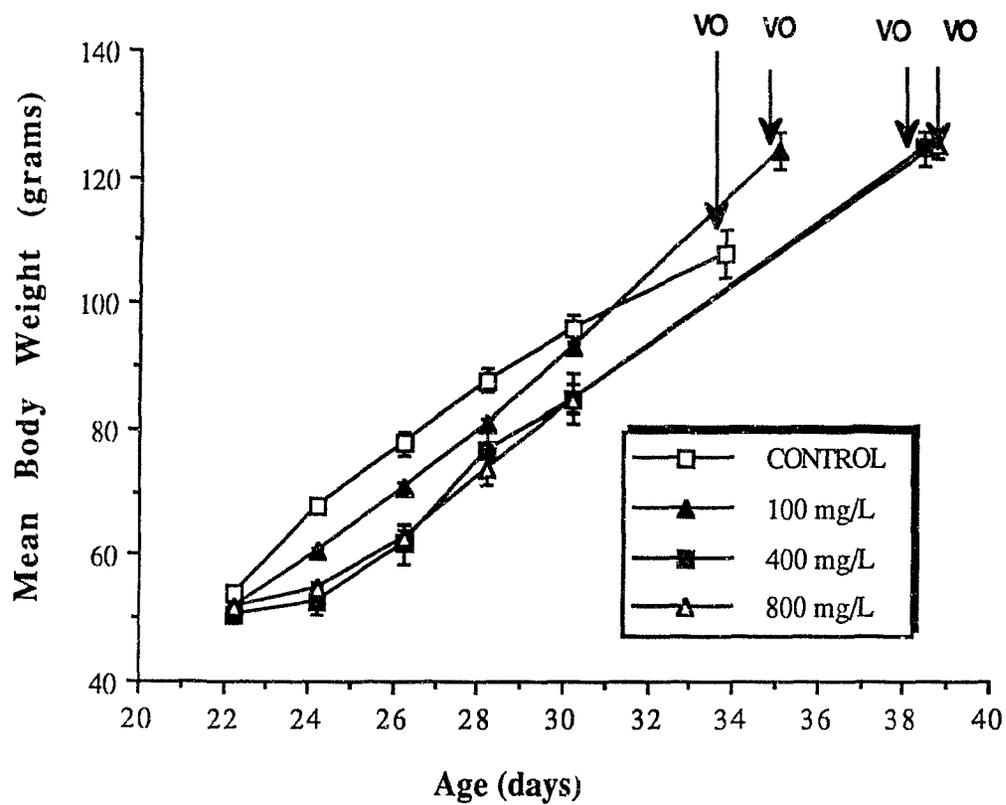


Fig. 33. Effect of morphine sulphate in the drinking water beginning on day 22 on growth rate of prepubertal female rats. The mean day of V.O. and mean body weight at V.O. are indicated with arrows. Values are means  $\pm$  s.e.m.

days after V.O. even though morphine was still being administered in the drinking water. Rats that were not delayed in diestrus cycled normally.

## 2. Effects of Morphine on Adult F      Cyclicality

A similar experiment, in which the final concentration of morphine was 800 mg/L (stepped up from 400 mg/L), was performed on adult, morphine-naive rats that had exhibited 2 consecutive estrous cycles (4 days/cycle). Figure 34 demonstrates that at this concentration of morphine, 100% of the rats remained in diestrus for approximately 10 days of opiate treatment at which time, all rats spontaneously began to cycle even though morphine treatment was continued. Increasing the concentration of morphine to 800 mg/L (9 days) did not affect cyclicality.

## 3. Effect of Fentanyl on the Timing of V.O.

Preliminary experiments, in which the potent mu-agonist fentanyl citrate was dissolved in the drinking water, were performed without the addition of sucrose. In the absence of sucrose to mask the bitter taste of the drug, water and fentanyl consumption decreased. The reduced fentanyl intake probably accounts for the lack of effect of fentanyl treatment on V.O. Also noted was the absence of withdrawal symptoms exhibited when injected with naloxone. Thus, all subsequent experiments were performed with the addition of 50 g/L of sucrose to the water.

Based on a 1.5% bioavailability (Schneider and Brune, 1985) and a fluid intake of 250 ml/kg/day, animals administered 4.8, 9.6, 19.2

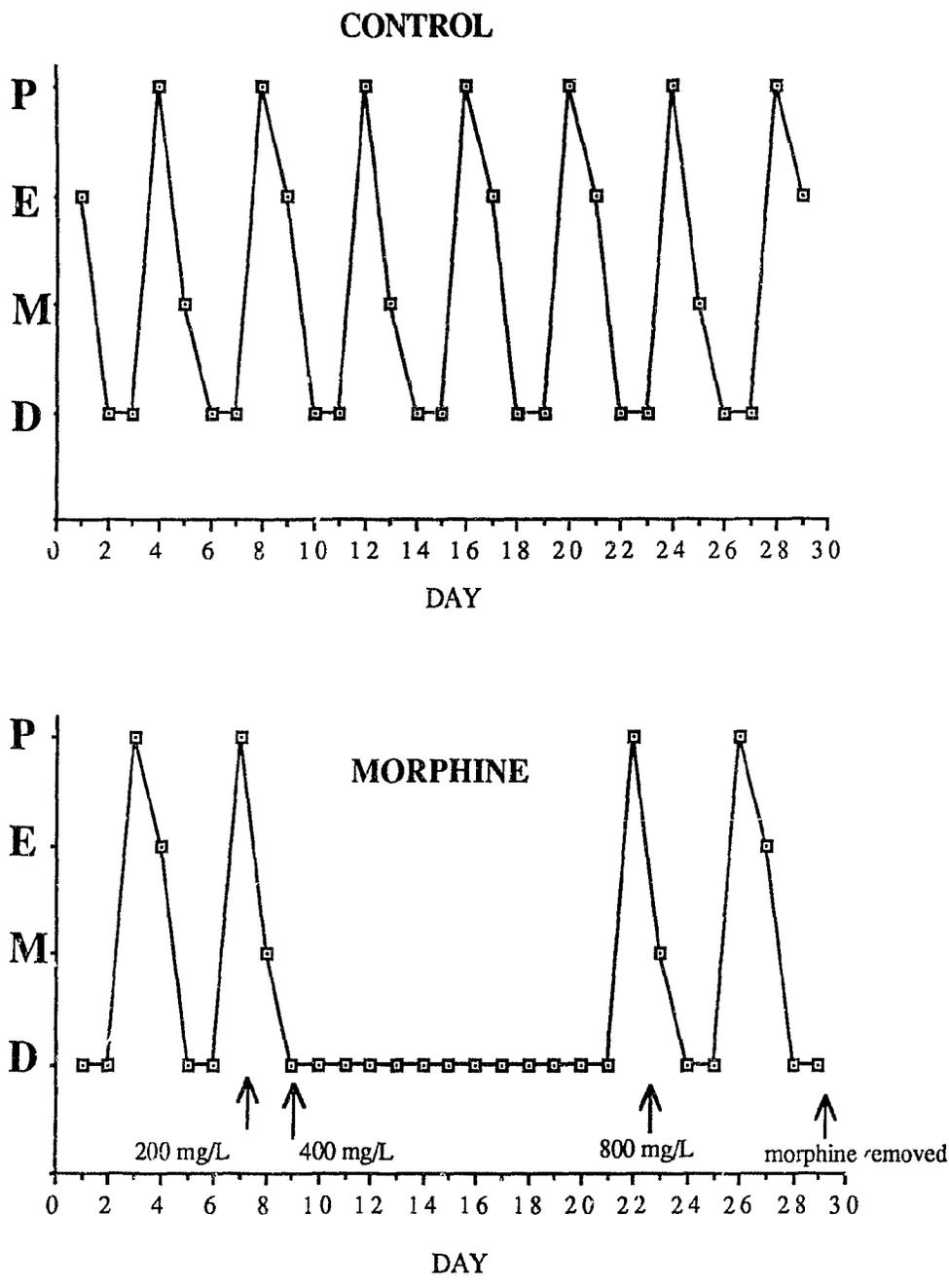


Fig. 34. Effect of morphine sulphate dissolved in the drinking water on adult estrous cyclicity. The top graph represents a control estrous cycle whereas the one below represents an estrous cycle disturbed by morphine treatment. The letters on the y-axis represent the four stages of the estrous cycle. P: proestrus; E: estrus; M: metestrus; D: diestrus.

and 30 mg/L of fentanyl citrate received 0.018, 0.036, 0.072 and 0.112 mg/kg/day respectively. Oral administration of fentanyl citrate at the higher concentrations, 19.2 and 30 mg/L, significantly delayed V.O. as shown in Figure 35. The growth curves (Fig. 36) of fentanyl- treated rats were not significantly different from controls and all treated rats, except for the 9.6 mg/L group, were significantly heavier at V.O. than controls (Table 12). The number of rats that had ovulated on the day of V.O. were: Controls 100% (10/10); 4.8 mg/L group 80% (8/10); 9.6 mg/L group 80% (8/10); 19.2 mg/L group 60% (6/10); 30 mg/L group 50% (5/10). All rats that did not ovulate were in proestrus. In a similar experiment animals were not sacrificed at V.O but had wet vaginal smears taken twice daily for 12 days. Unlike morphine treated animals, rats (n=5) from each group cycled normally (4 days/cycle).

#### 4. Effect of Fentanyl on Adult Estrous Cyclicity

When fentanyl-naive adult female SD rats were administered 30 mg/L of fentanyl citrate (stepped up from 20 mg/L) in their drinking water, they did not become acyclic nor did they cycle irregularly. When the concentration of fentanyl was then stepped up to 40 mg/L (Fig 37), 33% (2/6) became acyclic (remained in diestrus); 17% (1/6) remained in diestrus for 6 days and then began to cycle; and 50% (3/6) cycled normally.

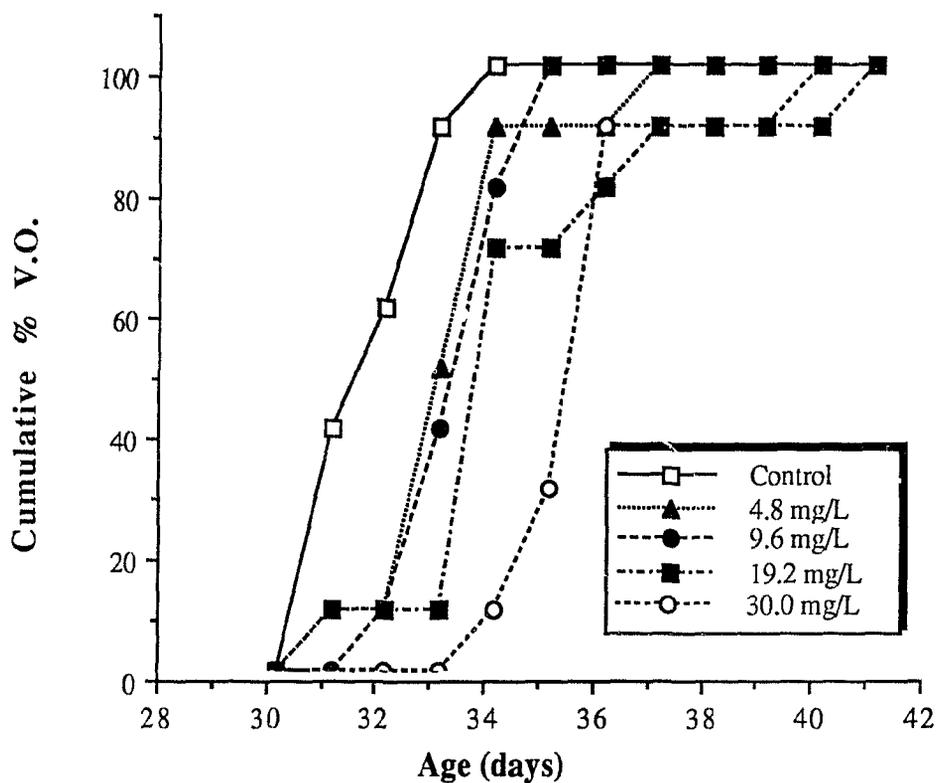


Fig. 35. Effect of different concentrations of fentanyl citrate (4.8 mg/L; 9.6 mg/L; 19.2 mg/L; and 30 mg/L; from day 22 until V.O.;  $n=10$ /treatment) dissolved in acidified and sweetened drinking water, on the timing of V.O. Controls ( $n=10$ ) received acidified and sweetened drinking water. Data plotted as the cumulative % of rats showing V.O. at each age.

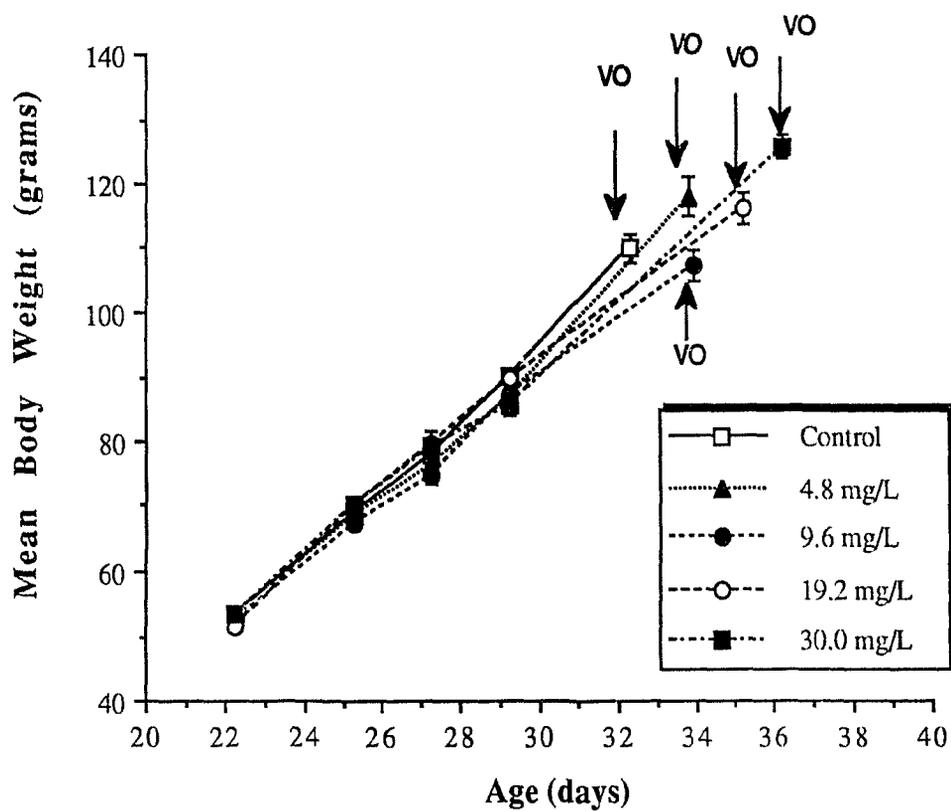


Fig. 36. Effect of different concentrations of fentanyl citrate administered via the drinking water beginning on day 22, on growth rate of prepubertal female rats. The mean day of V.O. and mean body weight at V.O. are indicated with arrows. See Table 12 for details. Values are means  $\pm$  s.e.m.

**Table 12.** The effect of fentanyl citrate via the drinking water on the age at V.O. in immature female rats.

	n	Age (days)	Body Wt. (grams)	Uterus (mg/100 g B.W)	Ovaries (mg/100 g B.W)	Ant. Pit (mg/100 g B.W.)
CONTROL	10	32.1±0.3	108.4±2.3	145.3±9.2	28.7±1.6	3.9±0.1
Fentanyl (4.8 mg/L)	10	33.6±0.4	116.5±3.1*	127.5±13.0	28.1±2.1	3.8±0.2
Fentanyl (9.6 mg/L)	10	33.7±0.3	105.9±2.5	159.1±10.7	30.1±1.4	3.9±0.2
Fentanyl (19.2 mg/L)	10	35.0±0.8**	116.2±2.7*	134.2±11.7	30.4±1.9	3.6±0.2
Fentanyl (30 mg/L)	10	36.0±0.5**	124.2±1.8**	110±10.7*	24.1±1.9*	3.5±0.3

Values are mean± s.e.m.\* p<0.05 ; \*\* p<0.01 compared to Controls; ANOVA and post hoc Dunnett t-test

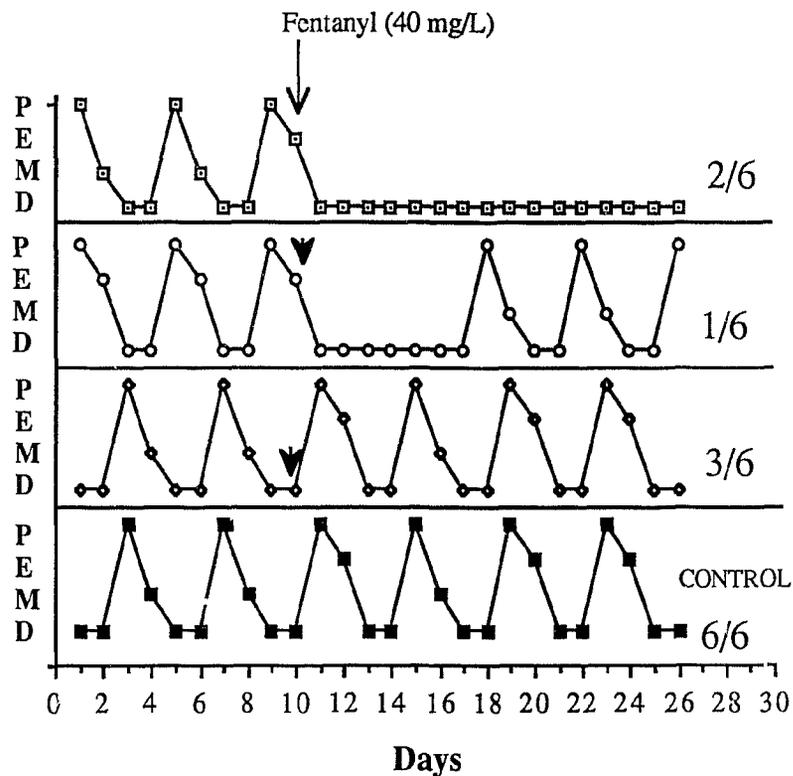


Fig. 37. Effect of fentanyl citrate dissolved in the drinking water on adult estrous cyclicity. All groups ( $n=6$ ) (except controls;  $n=6$ ) had been treated with 30 mg/L of fentanyl before the dose was increased to 40 mg/L. The letters on the y-axis represent the four stages of the estrous cycle. P: proestrus; E: estrus; M: metestrus; D: diestrus. The numbers beside each cycle (i.e. 1/6) represents the number of rats demonstrating that particular pattern of estrous cyclicity.

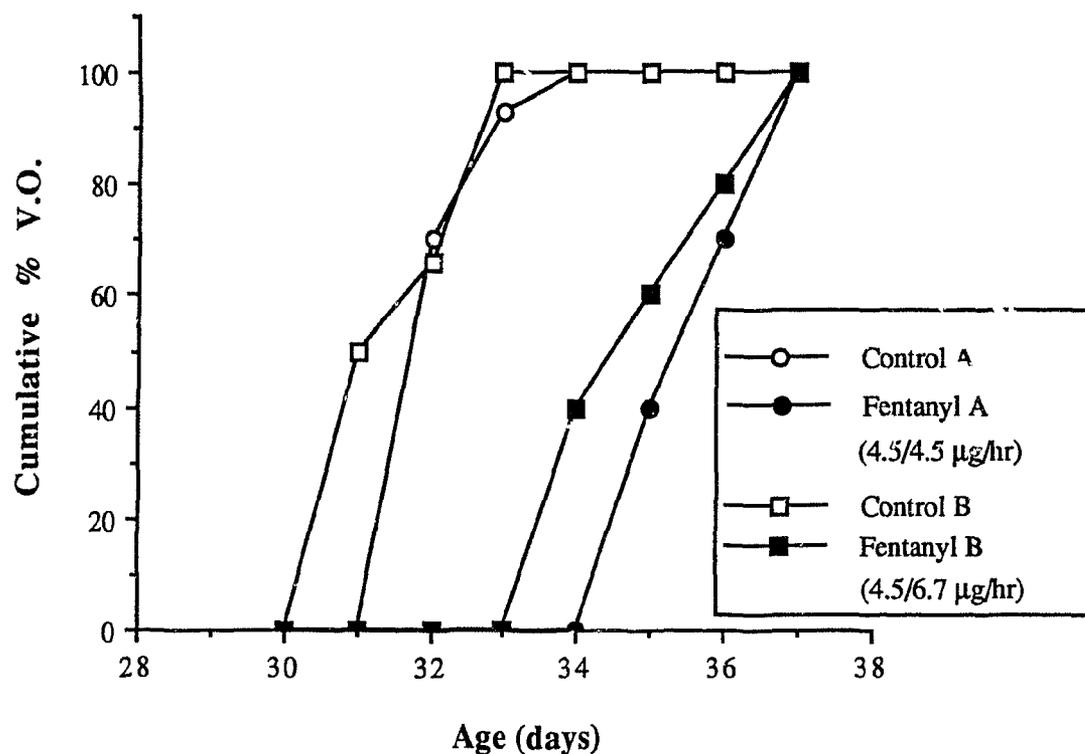
### 5. Effect of Fentanyl, via Osmotic Minipumps (2 x 7 day pumps), on the Timing of V.O.

The concentrations of fentanyl placed in the pumps was estimated from the bioavailable dose used in the drinking water i.e. a concentration of 30 mg/L of fentanyl in the drinking water, was equivalent to a dose of 0.112 mg/kg/day (or 4.7  $\mu\text{g}/\text{kg}/\text{hr}$ ). The bioavailability of fentanyl, released subcutaneously, was unobtainable. The cost of osmotic pumps did not allow the determination of optimal doses of fentanyl. However, pumps which delivered 1.8 mg/kg/day were effective in delaying the timing of V.O.

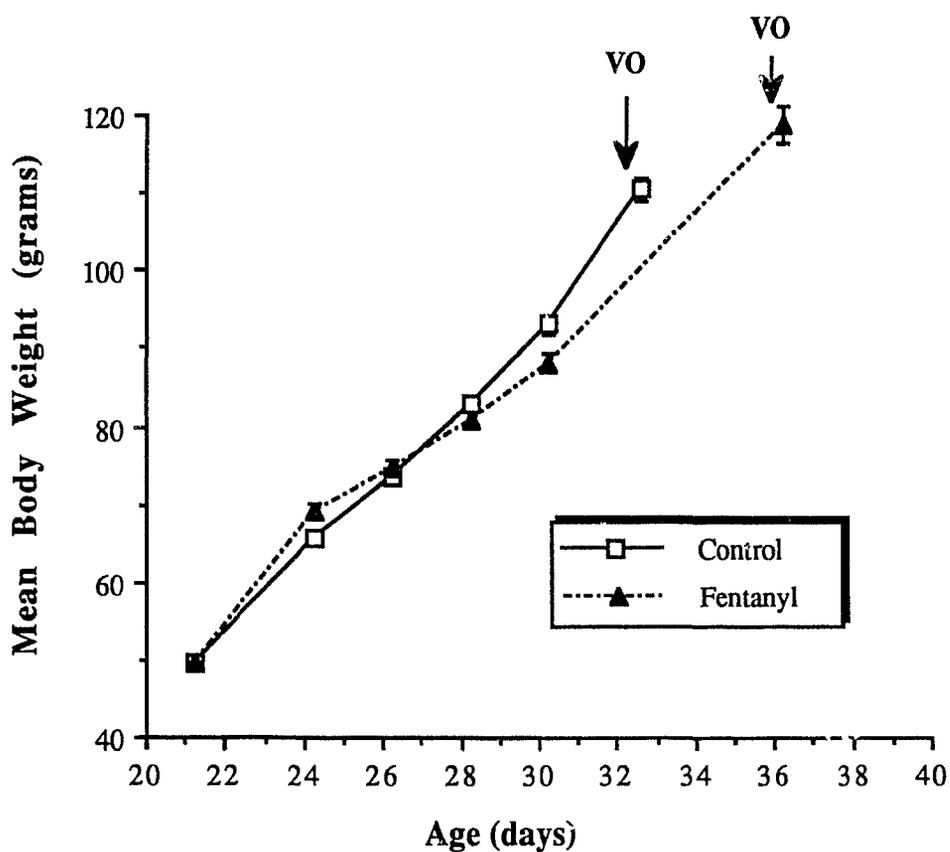
Figure 38 illustrates the results of two experiments. In the first (treatment A), minipumps delivering 4.5  $\mu\text{g}/\text{hr}$  (1.8 mg/kg/day) of fentanyl citrate, implanted on day 24 and replaced on day 31 (4.5  $\mu\text{g}/\text{hr}$ ) resulted in a delay of V.O. This dose of fentanyl did not markedly affect growth rate (fig 39). At V.O., the treated group had a mean body weight that was significantly greater than control values (Table 13). On the day of V.O., 100% of the rats from each group ovulated. Interestingly the mean uterine weight of the treated group was significantly smaller when compared to controls while the ovarian weight was significantly greater than controls.

### 6. Effect of Fentanyl via Osmotic Minipump (14-day pump) on the Timing of V.O.

In this experiment (treatment B), rats had pumps implanted on day 24 (4.5  $\mu\text{g}/\text{hr}$ ) as previously described (above) except that on day 31 the dose of fentanyl placed in the pumps was increased (6.7



**Fig. 38.** Effect of fentanyl citrate administered via osmotic minipumps (from day 24 until V.O.) on the timing of V.O. Treatment A: pumps implanted on day 24 and day 31 delivered 4.5  $\mu\text{g/hr}$  ( $n=10$ ). Treatment B: pumps implanted on day 24 delivered 4.5  $\mu\text{g/hr}$ ; pumps implanted on day 31 delivered 6.7  $\mu\text{g/hr}$  ( $n=5$ ). Controls from each experiment (A;  $n=10$  and B;  $n=5$ ) were surgically prepared but did not receive pumps. Data plotted as the cumulative % of rats showing V.O.



**Fig. 39.** Effect of fentanyl citrate administered via osmotic minipumps ( $4.5 \mu\text{g/hr}$ ; Treatment A) on the growth rate of prepubertal female rats. Treatment B (not shown) provided identical results. The mean day of V.O. and mean body weight at V.O. ( $\pm$ s.e.m.) are indicated with arrows. See Table 13 for details.

**Table 13.** The effect of fentanyl citrate (4.5/4.5  $\mu\text{g/hr}$  or 4.5/6.7 $\mu\text{g/hr}$ ) via osmotic minipumps on the age at V.O in prepubertal female rats.

	n	Age (days)	Body Wt. (grams)	Uterus (mg/100 g B.W)	Ovaries (mg/100 g B.W.)	Ant. Pit. (mg/100 g B.W.)
1. Control	10	32.4 $\pm$ 0.2	109.3 $\pm$ 1.6	153.4 $\pm$ 4.8	30.8 $\pm$ 1.5	3.6 $\pm$ 0.3
Fentanyl (4.5/4.5 $\mu\text{g/hr}$ )	10	35.9 $\pm$ 0.3**	117.6 $\pm$ 2.4*	124.2 $\pm$ 6.7*	33.5 $\pm$ 1.5*	3.5 $\pm$ 0.2
2. Control	5	31.6 $\pm$ 0.4	101.6 $\pm$ 1.5	---	---	---
Fentanyl (4.5/6.7 $\mu\text{g/hr}$ )	5	35.2 $\pm$ 0.6**	115.7 $\pm$ 3.6**	---	---	---

Values are mean  $\pm$  s.e.m. \*  $p < 0.05$ ; \*\*  $p < 0.005$  compared to respective Controls; Student's t-test

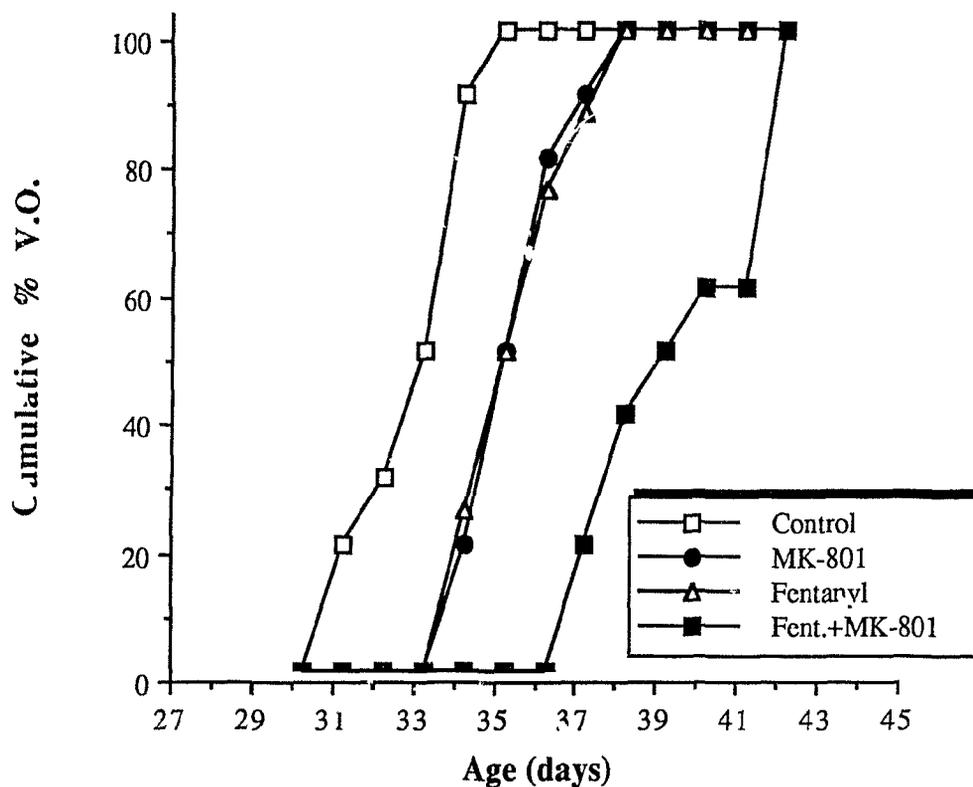
$\mu\text{g/hr}$ ; 1.8 mg/kg/day) to compensate for the increase in body weight. This procedure resulted in a similar delay of V.O. (Fig 38; Table 13) Again, when compared to controls, growth rate was unaffected (data not shown) and on the day of V.O. the treated group had a mean body weight that was significantly greater than the control weight (Table 13). Vaginal smears, taken twice daily immediately after V.O., indicated that animals receiving the increased dose of fentanyl cycled regularly (4 days/cycle).

#### 7. Effect of Fentanyl via Osmotic Minipumps (14-day) on Adult Estrous Cyclicity

Daily wet vaginal smears taken from regularly cycling adult female rats implanted with fentanyl-filled osmotic minipumps ( $15\mu\text{g/hr}$ ), indicated that estrous cyclicity was unaffected by continuous administration of this potent mu-agonist (data not shown).

#### 8 Effect of Fentanyl via Osmotic Minipumps (14-day pump) and MK-801 Injections on the Timing of V.O.

The combined effects of fentanyl via osmotic pump and injection of the glutamate antagonist MK-801 are illustrated in Fig. 40. As previously demonstrated, MK-801 or fentanyl alone (see Fig 6 and Fig. 35 respectively) significantly delayed the timing of V.O. The body weights of the treated groups were also significantly heavier than controls (Table 14). The combination of the two drugs, however, resulted in a delay of V.O. that was over twice that of either drug used separately. Body weights were significantly heavier than controls or groups receiving one treatment. Ovarian weights and the



**Fig. 40.** Effect of fentanyl citrate ( $4.5 \mu\text{g/hr}$ ; implanted on day 24) via osmotic mini-pumps and co-injection of MK-801 ( $0.1 \text{ mg/kg s.c.}$ ; beginning on postnatal day 27) on the timing of V.O. Data plotted as the cumulative % of rats showing V.O. at each age.

**Table 14.** Effects of fentanyl citrate (via osmotic minipumps; implanted on postnatal day 24) and s.c. injections of MK-801 (beginning on postnatal day 27) on age, body weight, organ weight and number of corpora lutea (C.L.) at vaginal opening. Values are mean $\pm$ s.e.m.

	Control	Fentanyl (4.5 $\mu$ g/hr)	MK-801 (0.1 mg/kg)	Fentanyl+ MK-801
n (rats/group)	10	10	10	10
Age at V.O. (days)	33.1 $\pm$ 0.4	35.6 $\pm$ 0.5**	35.6 $\pm$ 0.4**	39.4 $\pm$ 0.7**,††
Body Wt. (g)	108.3 $\pm$ 2.8	118.1 $\pm$ 2.4**	118.2 $\pm$ 2.8*	127.7 $\pm$ 3.9**,†
Paired Ovarian Wt. (mg/100 g B.W)	32.3 $\pm$ 2.1	29.3 $\pm$ 1.7	29.6 $\pm$ 1.3	28.8 $\pm$ 1.2
Total CL	10.1 $\pm$ 0.2	10.5 $\pm$ 0.2	10.8 $\pm$ 0.4	11.0 $\pm$ 0.5

\* p<0.05; \*\* p<0.005 when compared to controls. ANOVA and post hoc Dunnett t-test† p<0.05 when compared to fentanyl and MK-801-treated rats. and†† p<0.005 when compared to fentanyl and MK-801-treated rats using ANOVA and post hoc Scheffe F-test..

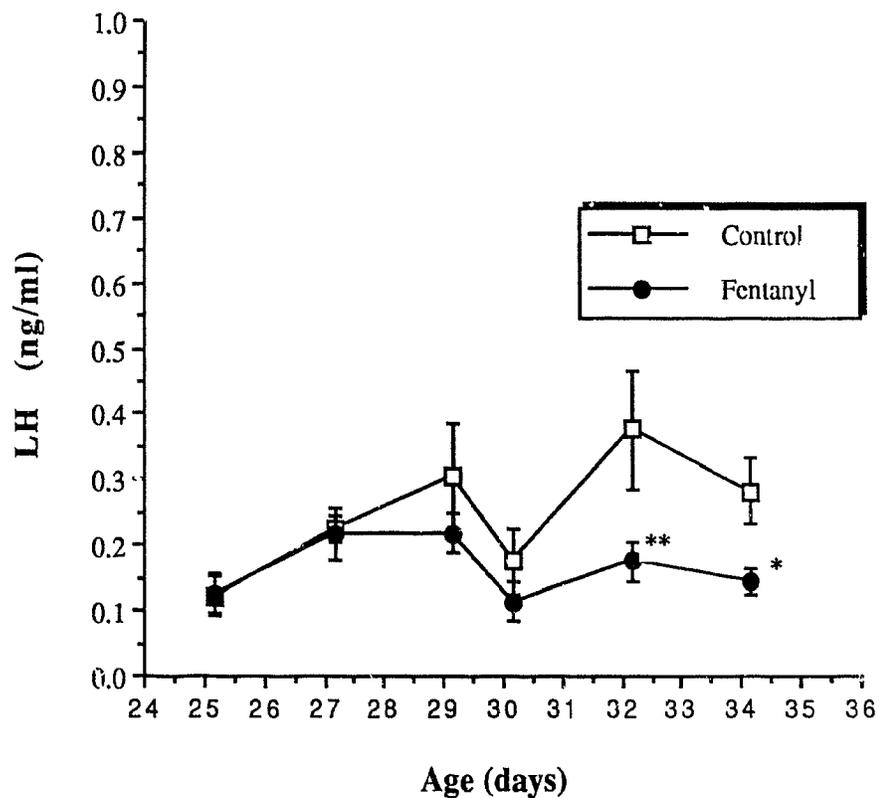
number of C.L. at V.O. were unaffected by any of the drug treatments (Table 14).

#### 9. Effects of Fentanyl via the Drinking Water on Basal LH Secretion prior to V.O.

The effects of fentanyl on basal LH secretion prior to V.O. are demonstrated in Fig. 41. LH serum concentrations were not significantly different between control and fentanyl-treated rats from postnatal day 25 to postnatal day 30. However, at 30 days of age LH concentrations in control rats begins to increase whereas in fentanyl-treated rats, LH concentrations remain low (Con:  $0.36 \pm 0.09$  vs Fent:  $0.16 \pm 0.03$  ng/ml;  $p < 0.025$ ). On postnatal day 34 LH levels were still significantly greater in control rats when compared to fentanyl-treated rats (Con:  $0.26 \pm 0.05$  vs Fent:  $0.13 \pm 0.02$  ng/ml;  $p < 0.05$ ). It should be noted that the majority of controls were in proestrus at 34 days of age, although all rats were sacrificed before 11:00 am to avoid interference of the proestrus LH surge. Table 15 indicates that the differences in body weight between postnatal days 25-34 were never significantly different between the control and treated groups. Reproductive organs however, often weighed significantly less in fentanyl-treated rats, especially uterine and anterior pituitary weights, and appeared to parallel the decrease in LH secretion in these rats.

#### 10. Effect of Naloxone on Morphine and Fentanyl-treated Rats.

When animals were injected with naloxone, a variety of withdrawal signs were observed that varied in number and



**Fig. 41.** Effect of fentanyl citrate via the drinking water (30 mg/L) on LH secretion between postnatal days 25-34. Rats used on days 32 and 34 had not experienced V.O. \* $p < 0.05$ ; \*\* $p < 0.025$  when compared to respective controls. Values are mean  $\pm$  s.e.m. (n=9-10 rats per group)

**Table 15.** Effect of fentanyl citrate via the drinking water (30 mg/L) on reproductive organ weights† prior to V.O.

Day	n	Body Weight (grams)	Ovaries (mg/pair)	Uterus (mg)	Ant. Pit. (mg)
<u>Day 25</u>					
Control	10	66.1±1.5	19.1±0.6	30.8±1.4	2.8±0.04
Fentanyl	10	71.0±1.5	17.1±0.7*	27.2±1.3*	2.7±0.08
<u>Day 27</u>					
Control	10	80.0±1.8	22.6±1.2	42.4±3.2	3.3±0.06
Fentanyl	10	81.3±1.9	23.2±1.4	32.4±2.2*	2.8±0.08**
<u>Day 29</u>					
Control	10	89.5±1.6	21.0±0.7	48.7±2.6	3.3±0.1
Fentanyl	10	87.1±1.4	20.6±1.3	41.4±4.4	3.5±0.06
<u>Day 30</u>					
Control	9	94.5±1.6	21.1±0.9	55.9±4.1	3.8±0.1
Fentanyl	10	95.4±2.8	23.0±1.0	56.9±5.5	3.3±0.1**
<u>Day 32</u>					
Control	9	103.0±2.4	22.6±1.1	68.6±8.8	4.2±0.2
Fentanyl	10	100.6±1.5	21.8±0.8	54.2±3.5	3.3±0.1**
<u>Day 34</u>					
Control	10	121.2±2.2	31.8±3.2	92.4±6.0	5.4±0.2
Fentanyl	10	116.1±3.0	22.1±1.0***	62.4±4.3***	3.9±0.1***

Values are  $\pm$ s.e.m. \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.005 when compared to respective controls; Student's t-test. † Absolute weights reported; when converted to per 100 g B.W., level of significance was unaffected.

intensity, usually increasing as the drug concentration increased (Table 16). Of the behavioral parameters tested, diarrhea was the only symptom seen in 100% of the animals in each of the experimental groups. It is also noteworthy that diarrhea was always seen within the first 5 min of the naloxone injection and was usually followed quickly by continuous chewing and wet dog shakes. The withdrawal signs observed in rats with osmotic minipumps (data not shown) were identical to the animals that were administered 30 mg/L of fentanyl in their drinking water.

**Table 16.** Naloxone (2.5 mg/kg)-induced withdrawal symptoms in morphine- and fentanyl-treated rats

Behavioral Parameters	Morphine sulphate				Fentanyl citrate			
	CON	100 mg/L	400 mg/L	800 mg/L	4.8 mg/L	9.6 mg/L	19.2 mg/L	30 mg/L
Wet Dog Shakes	2 / 5	4 / 5	5 / 5	5 / 5	2 / 5	4 / 5	5 / 5	5 / 5
Diarrhea	0 / 5	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5	5 / 5
Teeth Chattering	1 / 5	4 / 5	5 / 5	5 / 5	2 / 5	3 / 5	5 / 5	5 / 5
Chewing	1 / 5	4 / 5	5 / 5	5 / 5	3 / 5	4 / 5	5 / 5	5 / 5
Writhing	0 / 5	2 / 5	2 / 5	4 / 5	1 / 5	2 / 5	2 / 5	4 / 5
Ptosis	0 / 5	2 / 5	2 / 5	4 / 5	1 / 5	2 / 5	3 / 5	4 / 5
Irritability to:								
Touch	0 / 5	2 / 5	5 / 5	5 / 5	1 / 5	2 / 5	5 / 5	5 / 5
Handling	0 / 5	3 / 5	5 / 5	5 / 5	2 / 5	3 / 5	5 / 5	5 / 5
Jumping	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5	0 / 5

## Discussion

These experiments, based on the reliable bioassay of V.O. and first ovulation demonstrate that the induction of opiate dependence via chronic morphine administration does not prevent sexual maturation in female rats. Similar treatment with morphine but not fentanyl, in adult cyclic rats completely blocked estrous cyclicity. Previous studies on the effects of morphine on the age of V.O. were inconclusive, primarily because of the widely different drug schedules employed by these investigators. In these experiments, a technique designed to produce morphine dependence as described by Leung et al. (1986ab) was used. Earlier work by this group and others (Badawy et al., 1982; Van der Laan et al. 1988) have shown that the induction of opiate dependence via the oral route is simple, quick, and minimizes stress in experimental animals, a factor that is critical when examining sexual maturation in rats. The actual doses used here, based upon bioavailability (see Results from this Sect.), were relatively small i.e. for morphine: range, 4-32 mg/kg/day; for fentanyl: range, 0.018-0.112 mg/kg/day. Recent work by Cicero et al. (1989), in which morphine pellets (75 mg) were implanted in the immature male rat, demonstrated a profound effect of the implants on the development of the reproductive system. The results from experiments presented here, demonstrated no such effect on the reproductive system in the female. It is possible that prepubertal male rats are much more sensitive to morphine than prepubertal females (Cicero et al. 1986), although it is difficult to compare morphine doses between the two studies. At the time of morphine

pellet implantation one can estimate the dose to be 1071 mg/kg (assuming a body weight of 70 g). The highest dose of morphine used here (800 mg/L in drinking water) translates to 200 mg/kg/day (oral; equivalent to 32 mg/kg/day assuming a bioavailability of 20 %). Thus, the techniques of oral intake or minipump application suggest themselves to be a more sensitive and versatile approach to the study of opiate regulation of sexual maturation.

By administering opiates in the drinking water, a diurnal pattern of drug intake (in rodents in which fluid consumption is increased when the lights are out and decreased when the lights are on (Gordon et al. 1986) has been achieved. Thus, the effects of a discontinuous but chronic opiate intake have been examined. Morphine administered in this manner significantly delayed the day of V.O. Remarkably, more than 80% of the drug-treated rats ovulated at this time, in spite of continued morphine treatment. The effects of morphine on growth are similar to those previously seen by other investigators using different modes of administration (Weiner and Scapagnini 1974; Zimmerman et al. 1974; Van der Laan et al. 1988). When animals initially received morphine, growth rate briefly decreased but once habituation was complete, the rate of weight gain was similar to controls and on the day of VO, treated animals were significantly heavier. This suggests that V.O. is not delayed simply through a reduction in weight gain. Interestingly, the animals that continued to receive morphine after VO became acyclic for a short time even though first ovulation had occurred. Subsequently, while still receiving morphine, they began to cycle regularly. There is no ready explanation for these results although the development of tolerance

to the drug (i.e. in addition to dependence) may play a role in the reappearance of cyclicity. This result also implies that the mechanism of the first ovulation might be different from subsequent ones i.e. the second ovulation is more sensitive to the morphine dose which allowed the first ovulation to take place. It is noteworthy then, that when drug-naive adult female rats were treated with morphine, they immediately became acyclic and remained so for 10-11 days of treatment. After this time they began to cycle normally, even in the face of continued morphine intake. This could represent the appearance of tolerance, since increasing morphine intake (800 mg/L) did not effect cyclicity. However, since adult female rats do initially become acyclic this suggests that morphine clearance is not a factor in the occurrence of first ovulation (puberty) in morphine treated immature rats.

Similar experiments were conducted with the mu-agonist, fentanyl citrate, a drug reported to be 100-300 times more potent than morphine (Emmett-Oglesby et al. 1988; Jaffe and Martin 1985; Romagnoli 1973). Fentanyl, in contrast to morphine, did not significantly affect growth but did delay VO. Differences between the two drugs were apparent when given in large concentrations to adult female rats. Morphine prevented cyclicity in 100% of the rats, whereas fentanyl given as a near-lethal dose prevented only 30% of the animals from cycling. There are a number of possible reasons for this affect of fentanyl citrate. First, although fentanyl is more potent than morphine (Jaffe and Martin 1985) the bioavailability of fentanyl is much less than that of morphine (Schneider and Brune 1985). Secondly, although fentanyl has a high affinity for the mu

receptor, it has a much faster dissociation rate than morphine (Rigg and Goldsmith 1976) and therefore may not possess the same ability as morphine to block ovulation. The degree of dependence induced by either method was quantified by the number and severity of standard behavioral parameters exhibited over a 30 minute following injection with naloxone.

Is the delaying effect of morphine on the timing of V.O. due to the diurnal nature of the intake? It is possible that brain concentrations of morphine might remain high on a fairly continuous basis, particularly at the higher dose levels. There is some evidence that diurnal variations in the number of opioid receptors in the hypothalamus (Jacobson and Wilkinson 1986; Weiland and Wise 1987; Jacobson and Kalra 1989) and in whole brain (Wirz-Justice 1987) may contribute to the timing of cyclic physiological events. If so, then the opiate paradigm used here would likely derange any endogenous opiate receptor rhythm. On the other hand, if the brain is exposed to a diurnal rhythm in opiate binding (i.e. the water intake rhythm) it is not unreasonable to suggest that adaptation of receptor rhythms could take place, just as an animal might re-adjust to a new lighting regime for example.

As an alternative to discontinuous intake of opiate, osmotic minipumps were used to ensure continuous delivery. In preliminary studies, attempts were made to replicate the observations of Advis et al. (1982) who reported that immature female rats (day 23), implanted with osmotic minipumps delivering morphine at a rate of  $1\mu\text{g/hr}$ , reached V.O. 6 days later than controls. These attempts were unsuccessful because of solubility problems; i.e. sufficient morphine

based on the oral experiments could not be dispensed from the pumps (Morphine, released at a rate of 1  $\mu\text{g/hr}$ , did not delay the time of V.O.; results not shown). Instead fentanyl citrate was used, a concentrated solution of which could readily be placed in the minipumps. The results demonstrate that continuous delivery of fentanyl citrate delayed VO. just as observed in the oral experiments. However, post-V.O. estrous cyclicity was not prevented. This clearly implies that continuous, or discontinuous, opiate treatment is effective in slowing sexual maturation. Nevertheless, first ovulation and subsequent cyclicity were not prevented, and the neural drive to reach sexual maturation somehow negates opiate inhibition.

The role of glutamate and the NMDA receptor during sexual maturation was examined earlier in this Thesis. The results from those experiments implied that glutamate (NMDA) may be an excitatory influence that mediates the onset of puberty. Thus, eventual first ovulation observed in opiate-treated rats may be the result of increased NMDA receptor activation that "overcomes" opiate blockade and induces the LH surge. Previous experiments (see Sect. I Results p. 89) also demonstrated that NMDA-induced LH secretion may be mediated through norepinephrine release. This suggests that opioids and glutamate may be controlling GnRH/LH release through a common pathway. However, when rats were given a combination of fentanyl and the glutamate antagonist MK-801, the delay of V.O was greater than twice that of either treatment used alone. This suggests that glutamate, and EOP regulation of GnRH/LH secretion may be mediated through two distinct but converging pathways. Note however, that animals receiving both fentanyl and MK-801 did

eventually ovulate. The possibility that stimulation of non-NMDA receptors (i.e. quisqualate and kainate) by glutamate, may also have an excitatory influence on the onset of puberty in the female rat, cannot be overlooked. Recent studies by Lopez et al. (1990) have demonstrated that both NMDA and kainate receptors may be important regulators of GnRH secretion, although experiments examining the influence of each receptor type has on this system during sexual maturation are clearly needed.

Morphine and opioid peptides are known to block the proestrous surge of GnRH and subsequently LH secretion (Ching 1983) in rats (Bruni et al. 1977) as well as in monkeys (Ferin et al. 1983). In addition Cicero et al. (1988b; 1989) report that implanted morphine pellets profoundly inhibit secretion of LH for two weeks and reduce hypothalamic GnRH secretion for five to six weeks in immature male rats. When LH secretion was examined in prepubertal fentanyl-treated rats basal levels in control rats began to increase as puberty approached whereas LH levels in fentanyl-treated rats remained unchanged. Although these results suggest that fentanyl-induced decrease in LH secretion may have an important role in delaying V.O., they do not offer an explanation for eventual first ovulation observed in these rats. Measuring changes in serum LH concentrations until the afternoon of the first proestrous LH surge would certainly provide a better understanding of how female rats overcome continuous opiate blockade to achieve first ovulation. The problem encountered during this type of experiment and the ones presented here, is the uncertainty of the day of V.O.; i.e. rats cannot be used if they have ovulated. Using a large number of rats/group

may extend the number of days from which one can sample, but this is expensive and still may not provide information regarding changes in serum LH levels on the day of first ovulation. Ideally, experiments in which daily/hourly blood samples are removed from the same animal would provide the greatest insight into the hormonal changes occurring in fentanyl treated rats prior to first ovulation. Unfortunately the equipment required to perform such an experiment is not available in this laboratory. Thus, in the experiments presented here, the mechanism for eventual first ovulation- in the face of chronic exogenous opiate blockade- is difficult to define. It is conceivable that animals develop a tolerance to the inhibitory effects of morphine (or fentanyl) which then permits first ovulation. However, in rats treated with morphine at the higher doses (20 and 40 mg/kg/day), first ovulation was immediately followed by 6-8 days in diestrus. Normal cyclicity was then resumed even though morphine intake was continued. Thus, the putative state of tolerance, present at V.O. was quickly dispelled to reappear again 6-8 days later. As noted already (see above) this result infers that once ovulation has occurred, the hypothalamo-pituitary system quickly becomes re-sensitized to morphine. In contrast, the inhibitory effect of fentanyl is permanently lifted at the time of V.O.. One can only speculate on the mechanisms that might be involved here. For example, the steroidal milieu established at V.O. could conceivably interfere with, or counteract, the morphine tolerance, although this is not applicable to the effect of fentanyl. A direct influence of morphine/fentanyl on pituitary response to GnRH should also be considered. (see for example Blank et al. 1986),

although previous studies in the male rat emphasize that morphine has no direct effect either on the testes or the anterior pituitary (Millan and Herz 1985; Cicero 1987).

The results presented here suggest that the occurrence of first ovulation in normal, untreated rats could be due to the development of tolerance to endogenous opioid peptides. As sexual maturation approaches, gonadotropin secretion would 'escape' from opiate negative feedback. The experimental preparation used here appears to be analogous to that used by Foster and Ryan, (1979); i.e. estradiol-treated ovariectomized lambs exhibited marked increases in LH secretion coincident with puberty in intact lambs, even though the inhibitory levels of estradiol remained constant. Similar experiments have been performed in the immature rat (Steele and Weisz 1974).

Presented here is a simple preparation based upon a reliable bioassay (V.O.) with which to further investigate the role of EOP in pubertal development. The observation that immature female rats can reach first ovulation, even in the face of a continuous opiate blockade, raises interesting questions on the neurochemical regulation of sexual maturation.

## Section IV. DOWN-REGULATION OF OPIOID RECEPTORS *IN VITRO*

### Introduction

In the previous section the following question was posed: in the process of sexual maturation why do opiates/opioids become less effective in the inhibition of LH secretion? One possible approach is to investigate hypothalamic opioid receptors (binding sites). The diurnal variation observed in [<sup>3</sup>H]-NAL receptor density (Jacobson and Wilkinson 1986) suggests that the down-regulation of opioid receptors may play a critical role in the modulation of GnRH secretion during sexual maturation. However, many investigators have been unable to demonstrate a decrease in opiate receptor binding after chronic opiate agonist (i.e. morphine) treatment (for example Lenoir et al. 1983; Bardo et al. 1982; Bolger et al. 1988).

In an attempt to study the role and possible mechanism(s) of opioid receptor regulation, considerable effort has focused on the effects of exogenous opiates on a variety of opiate receptor populations. To date, studies devoted to agonist-induced down-regulation of opioid receptors have provided inconsistent results at best (Johnson and Fleming 1989; Loh et al. 1988; Tempel et al. 1988) perhaps due in part to the variety of tissue preparations and the number of different assay conditions used in these experiments. For example, receptor desensitization and down-regulation is readily induced by the delta agonist DADLE *in vitro*, using neuroblastoma x glioma NG108CC15 hybrid cells (Moses and Snell 1984) or

neuroblastoma N4TG2 cells (Blanchard et al. 1982; Chang et al. 1982). Unfortunately these cell lines contain a homogeneous population of the delta opioid receptor subtype and are therefore unsuitable for the study of the mu-subtype of opiate receptor. Although enkephalin-induced down-regulation of delta receptors has been demonstrated *in vivo* (Steece et al. 1986, 1989; Tao et al. 1988) similar studies examining mu-opiate receptors are less clear. Many groups, using whole brain homogenates, have reported that chronic morphine treatment *in vivo* fails to decrease mu-opiate receptor populations (Simon and Hiller 1978; Perry et al. 1982; Lenoir et al. 1983; Bardo et al. 1982; Bolger et al. 1988) whereas others have reported an increase in mu-receptor binding subsequent to continuous morphine treatment (Rothman et al. 1986; 1989; Lewis et al. 1984). Interestingly, acutely dispersed intact brain cells from rats chronically exposed to morphine and processed in physiological buffers resulted in a decrease in [<sup>3</sup>H]-naloxone binding without a change in receptor affinity ( $K_D$ ) (Rogers and El-Fakahany 1986). Similar experiments measuring the effects of chronic morphine treatment on guinea pig cerebrocortical membranes (Werling et al. 1989) and 7135C pituitary tumor cells maintained in primary culture (Puttfarcken et al. 1989) have been reported. Recently, Maloteaux et al. (1989) working with cultured neurons from rat forebrain, demonstrated that a number of potent agonists from the fentanyl series could induce mu-receptor down-regulation *in vitro*. This effect could not, however, be replicated with enkephalin derivatives or morphine.

These observations suggest that mu agonists are unlikely to decrease mu-receptor populations in a straightforward manner. This infers that opiate receptor down-regulation may not be a reasonable mechanism for the control of GnRH secretion during puberty. However, experiments in which metabolically active hypothalamic tissue punches (Wilkinson et al. 1990) have been used to study the regulation of cell surface opiate receptors *in vitro* are presented here and demonstrate that hypothalamic mu-receptor down-regulation can be achieved. This provides further support for the suggestion that opioid receptor down-regulation may be an important mechanism responsible for mediating GnRH release during sexual maturation, particularly during experiments in which rats are chronically exposed to opiate agonists (see Sect. III Results p. 155-159)

## Materials and Methods

### *Animals*

Female Sprague Dawley rats (Day 45-50) were obtained from Canadian Hybrid Farms, Halls Harbour, Nova Scotia, Canada. Animals were housed (3-4) in plastic cages under controlled lighting (lights on from 0700-2100h) and allowed free access to food and water. Rats were moved from the Animal Care Centre and allowed to settle down at least 1 hr before sacrifice.

### *Radioligands and Analogs*

[<sup>3</sup>H]-DAGO (S.A. 30.3-33.3 Ci/mmol) was obtained from New England Nuclear (Lachine, Quebec). Fentanyl citrate was generously provided by McNeil Laboratories (Canada) Ltd., Stouffville, Ontario. Unlabeled DAGO,  $\beta$ -endorphin and bacitracin were purchased from Sigma Chemical Company, St. Louis MO., U.S.A.

### *Brain Removal and Slicing*

Animals were sacrificed by decapitation between 10:00 hr and 11:00h on the day of the assay. Once the scalp was removed, the skull was cut coronally approximately 2 mm anterior to the olfactory bulbs. Scissors were inserted into the incision and used to cut through the skull at the lateral margins of the foramen magnum to the caudal end of the cerebellum. The dorsal surface of the skull was pulled back and the brain gently lifted to expose the optic nerves. The optic nerves were cut and a spatula carefully inserted underneath the ventral surface of the brain facilitating its removal from the cranial vault. Brains were placed in a beaker containing ice-

cold Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island NY) for approximately 10 min. When firm, the brains were placed on an inverted glass petri dish, on ice, with the ventral surface exposed. The hypothalamus was removed as a block by making an initial coronal cut just rostral to the optic chiasm and a second coronal cut just anterior to the mammillary bodies. The third and fourth cuts were made sagittally along the left and right lateral sulci. The hypothalamic block (approx. 3 mm deep) was removed and trimmed by making a horizontal cut at the anterior commissure. The block was then sliced coronally with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd. Comshell, Surrey) into 350  $\mu\text{m}$  slices. For preparation of hypothalamic micropunches, brain slices were separated with fine forceps and kept cold in a tissue culture dish (on ice) which had a cushion of Sylguard elastomer on the bottom, containing a minimal amount of buffer (DPBS). Using a stainless steel neuropunch, tissue punches (1.8 mm diam.) were removed from the arcuate-ventromedial hypothalamus and placed into a separate dish. The punches were distributed, one per well, into 24 well Linbro culture dishes (well capacity-3.5 mls) by carefully suspending each punch in a droplet of buffer using a pair of curved forceps. Leftover tissue punches (8-10) were weighed for determination of the mean wet weight/punch. The average weight for a 350  $\mu\text{m}$  thick (1.8 mm diameter) hypothalamic tissue punch was 1.00 mg  $\pm$  0.01 mg.

### *Down-regulation Assay*

Single hypothalamic punches were placed into individual wells of Linbro culture dishes containing 0.5 ml of DPBS and were allowed to preincubate at 37°C for 30 min. to remove any endogenous ligand that may occupy the receptors. After this preincubation, the DPBS was removed by Pasteur pipette and replaced with 0.5 ml of DPBS(+), (DPBS; HEPES (10mM); glucose (0.1%); H<sub>2</sub>O<sub>2</sub> (0.004%); Walton and Fulton 1983). Fentanyl citrate (10<sup>-5</sup> M), DAGO (10<sup>-6</sup>M or 10<sup>-5</sup>M) or β-endorphin (10<sup>-6</sup>M) used as ligands to down regulate mu-receptors, were pipetted (20 μl aliquots) into the wells. The punches were then incubated at 37°C for 120 min., the optimal time for μ-opioid receptor down-regulation (see below). After this incubation step, the buffer was removed and replaced with 0.5 ml of DPBS (warmed at 37°C) and incubated at 37°C for 5 min. This step was included to remove most of the free agonist. The DPBS was removed again and replaced with 0.5 ml of DPBS and incubated at 37°C for 30 min. This second wash removes any residual agonist that may occupy membrane receptor sites. Next, the DPBS was discarded and replaced with ice-cold 170 mM Tris-HCl (pH 7.4) buffer to facilitate mu-opioid receptor quantification using [<sup>3</sup>H]-DAGO ([D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>, glyo<sup>5</sup>] enkephalin; Wilkinson et al. , 37). Non-specific binding (NSB) was measured in the presence of unlabeled DAGO (10<sup>-6</sup>M) and was subtracted from the total bound counts to yield specific binding (SB). Both labeled and unlabeled drugs were added to the appropriate wells in aliquots of 20 μl, giving a final assay volume of 540 μl. Incubations were performed on ice for 4 hours in 170 mM Tris-HCl.

Incubation at this temperature prevents receptor internalization of the bound agonist ligand and also helps maintain tissue integrity.

Following incubation, 50  $\mu$ l samples (n=3) of buffer from each concentration were removed for determination of free (equilibrium) ligand concentration. The remaining buffer was removed with a Pasteur pipette and the tissue was washed with ice-cold Tris-HCl for 5 min. The punches were then removed with fragments of glass fibre filter paper (Whatman GF/B) and placed in counting vials containing scintillation cocktail (Formula 963, NEN). Radioactivity was quantified in a scintillation counter (LKB 1218 Rackbeta) at an efficiency of 40-44% for  $^3\text{H}$  (determined in the presence of the glass fibre). In this binding assay, 5-6 punches were normally used to determine total binding while 3-4 punches were used for determination of non-specific binding.

## Experiments

### 1. *Time Course of DAGO- and Fentanyl-Induced Mu-receptor Down-Regulation*

After the initial 30 min incubation at 37°C in DPBS, hypothalamic punches were incubated in DPBS+ with fentanyl ( $10^{-5}\text{M}$ ) or DAGO ( $10^{-6}\text{M}$ ) for 30, 60, 75, 90 or 120 min at 37°C. Mu-receptor binding was quantified with [ $^3\text{H}$ ]-DAGO (2.0 nM) as described.

### 2. *Effect of Agonist Concentration on Mu-receptor Down-Regulation*

Hypothalamic punches were incubated with increasing concentrations of fentanyl ( $10^{-8}$  -  $10^{-5}\text{M}$ ) or DAGO ( $10^{-7}$  -  $10^{-5}\text{M}$ ) at

37°C for 120 min. Mu-receptor binding was determined using [<sup>3</sup>H]-DAGO (1.5 nM).

### 3. *Quantification of Mu-receptor Down-Regulation with High and Low Concentrations of [<sup>3</sup>H]-DAGO.*

Based on the above experiments, hypothalamic tissue punches were preincubated at 37°C in DPBS for 30 min, followed by a 120 min incubation at 37°C in DPBS+ and DAGO (10<sup>-5</sup>M) or fentanyl (10<sup>-5</sup>M). Mu-receptor populations were initially assayed using concentrations of [<sup>3</sup>H]-DAGO between 1-8 nM. From these experiments it became obvious that a lower range of concentrations was more suitable, therefore subsequent studies used concentrations between 0.5-3 nM. (see Results and Discussion of this section).

### 4. *Effect of Bacitracin on Agonist-induced Mu-receptor Down-Regulation*

To determine whether the observed down-regulation of mu-opiate receptors was a result of receptor internalization via endocytosis, the anti-endocytotic agent bacitracin (2 mg/ml) (Maxfield et al. 1979; Davies et al. 1980) was added to the DPBS preincubation buffer (for control and drug-treated punches) for 30 min. Before incubation with the mu-agonists punches were rinsed with warm DPBS to remove excess bacitracin. The mu-opiate agonists DAGO (10<sup>-6</sup> or 10<sup>-5</sup>M) or fentanyl (10<sup>-5</sup>M) were then incubated with the tissue punches for 120 min and then assayed with [<sup>3</sup>H]-DAGO at two concentrations.

### *Data Analysis*

Saturation curves were analyzed, binding parameters (receptor density:  $B_{max}$ ; receptor affinity:  $K_D$ ) determined and curves fitted using GraphPAD InPlot Version 3.0 on an IBM PC. The  $SD_{erad}$  for all curves presented was less than or equal to 0.10 (Zivin and Waud 1982; computed on a Macintosh II)

### **Statistics**

Saturation curve analysis was based on a number of points combined from 3-10 experiments. Thus statistics performed on the binding parameters obtained from saturation curve analysis used the number of experiments performed to obtain a particular curve as the  $n$  value. Determination of significant differences between means was accomplished using Student's  $t$  -test with  $p < 0.05$  denoting a significant difference.

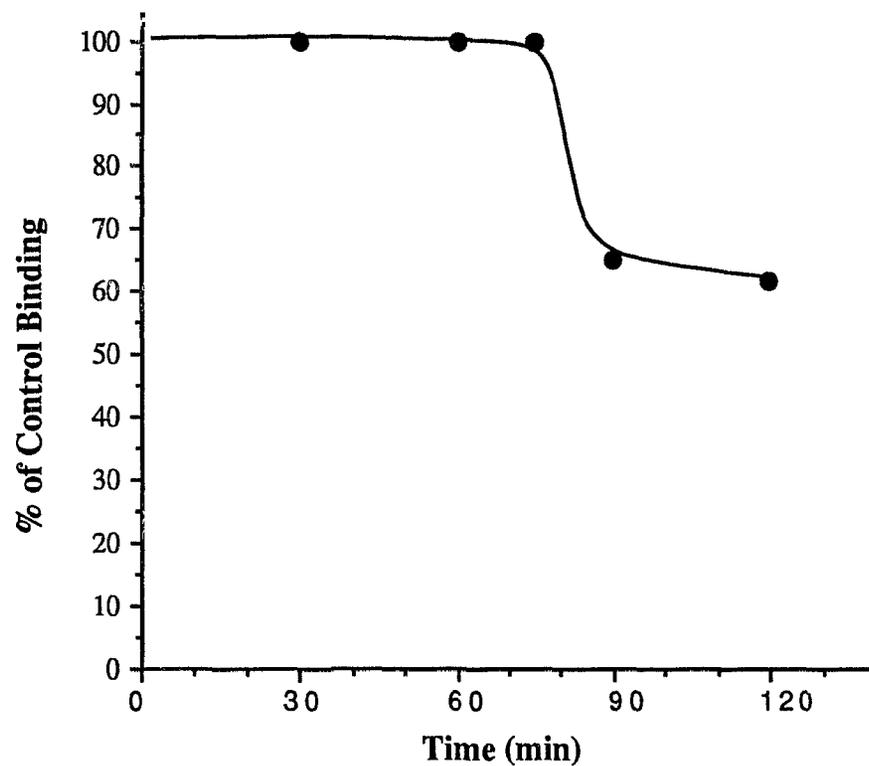
## Results

### 1. Time Course of DAGO- and Fentanyl- induced Mu-receptor Down-Regulation

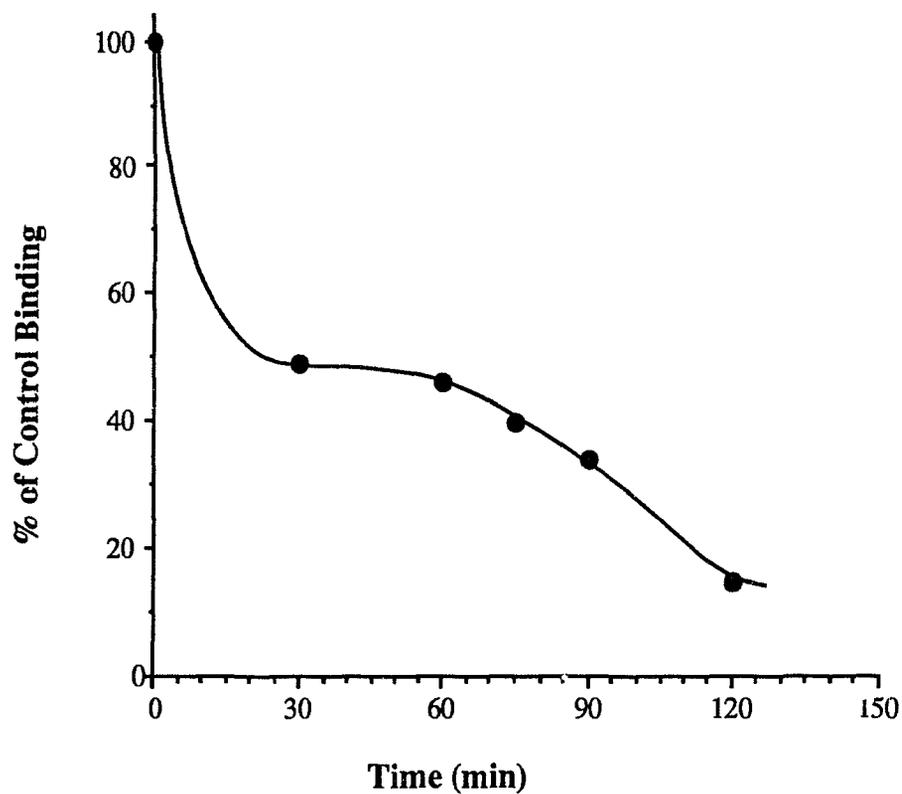
Tissue punches were preincubated with unlabeled DAGO ( $10^{-6}$ M) or fentanyl ( $10^{-5}$ M) for varying lengths of time and then assayed with [ $^3$ H]-DAGO (2 nM). The time course of DAGO-induced down-regulation (Fig. 42) expressed as % of control specific binding indicates that [ $^3$ H]-DAGO binding began to decrease after 75 min of DAGO preincubation, reaching 60% of control binding by 120 min. The fentanyl-induced decrease in specific [ $^3$ H]-DAGO binding, however, occurred after 30 min of exposure and was maximal by 120 min (Fig. 43). Incubations greater than 120 min at 37°C significantly reduced control binding (data not shown), thus all subsequent assays used an incubation time for down-regulation of 120 min.

### 2. Effect of Agonist Concentration on Mu-receptor Down-Regulation

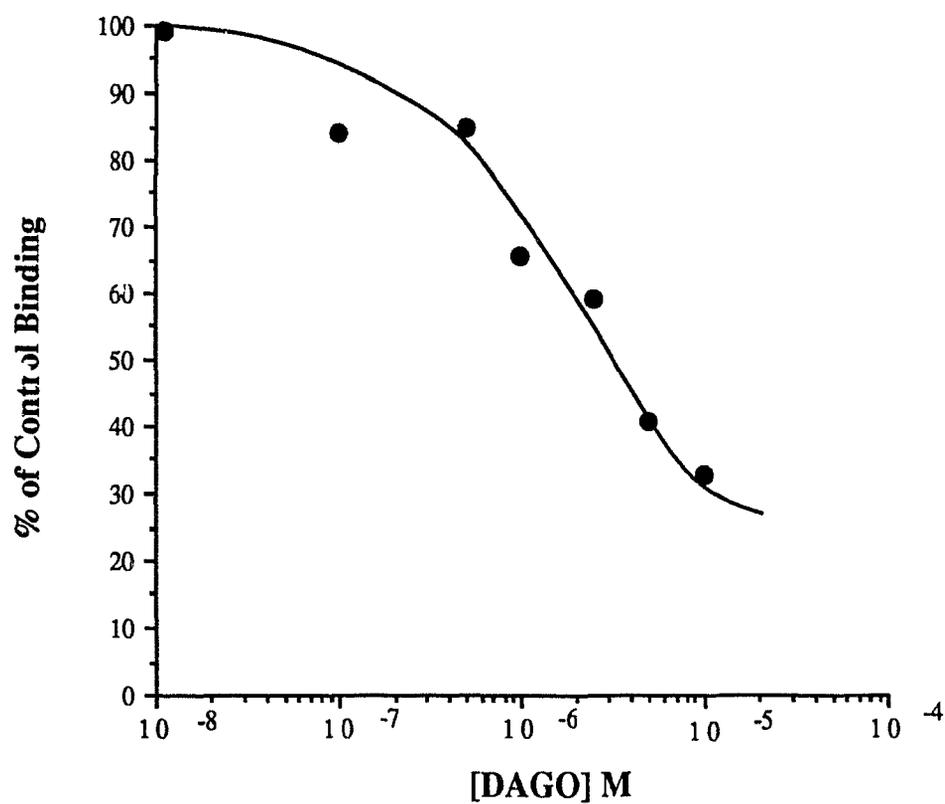
The effect of increasing concentrations of DAGO and fentanyl are illustrated in Fig. 44 and Fig 45. respectively. Although both agonists decrease specific [ $^3$ H]-DAGO binding by over 60% at  $10^{-5}$ M, DAGO (Fig. 44) appears to be more effective at decreasing binding at lower concentrations when compared to fentanyl. At a concentration of  $10^{-6}$ M, DAGO reduced [ $^3$ H]-DAGO binding to 63% of control whereas fentanyl only reduced binding to 89% of control.



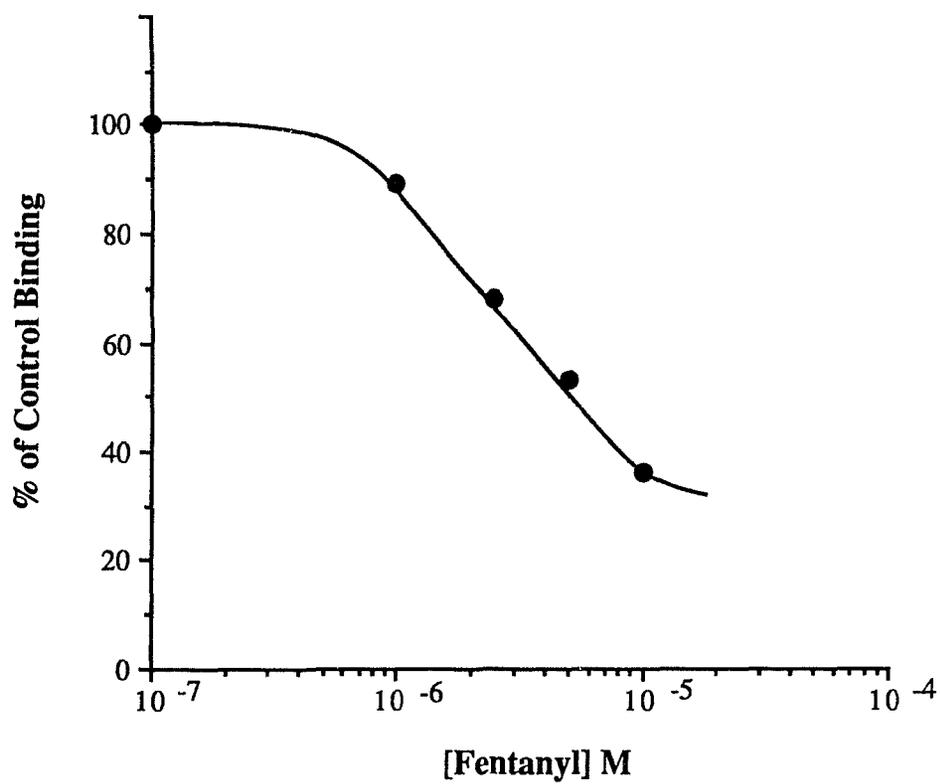
**Fig. 42.** Effect of incubation time on the DAGO ( $10^{-6}\text{M}$ )-induced decrease in specific [ $^3\text{H}$ ]-DAGO binding (2.0 nM) in hypothalamic tissue punches.



**Fig. 43.** Effect of incubation time on the fentanyl citrate ( $10^{-5}\text{M}$ )-induced decrease in specific [ $^3\text{H}$ ]-DAGO binding (2.0 nM) in hypothalamic tissue punches.



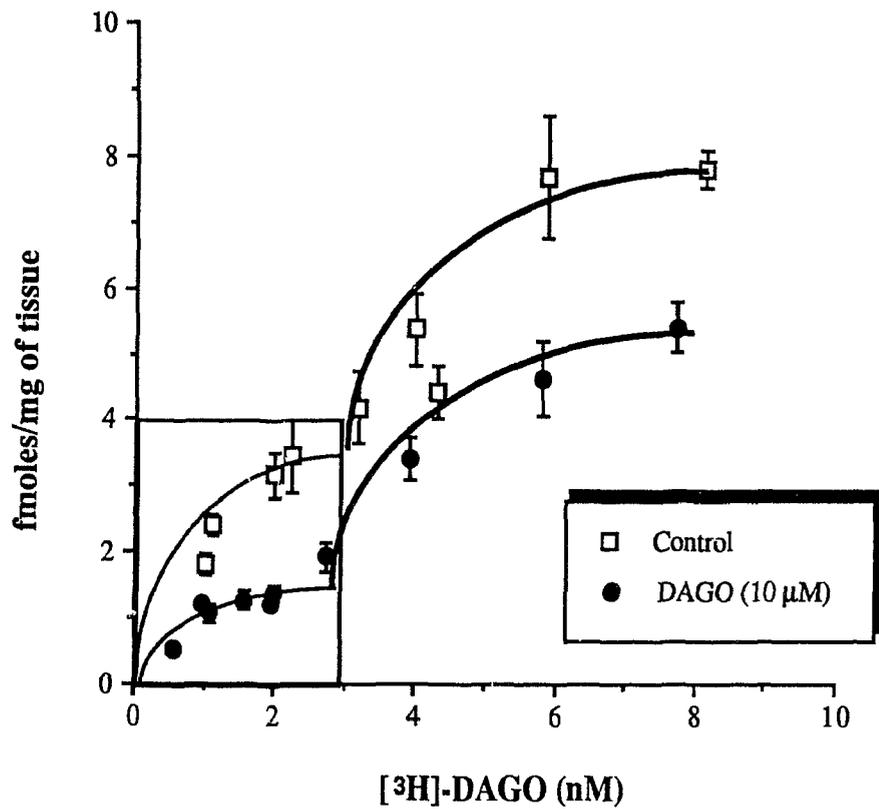
**Fig. 44.** Effect of incubation with different DAGO concentrations on specific [ $^3\text{H}$ ]-DAGO (1.5 nM) binding in hypothalamic tissue punches. Incubations were 120 min at  $37^\circ\text{C}$ .



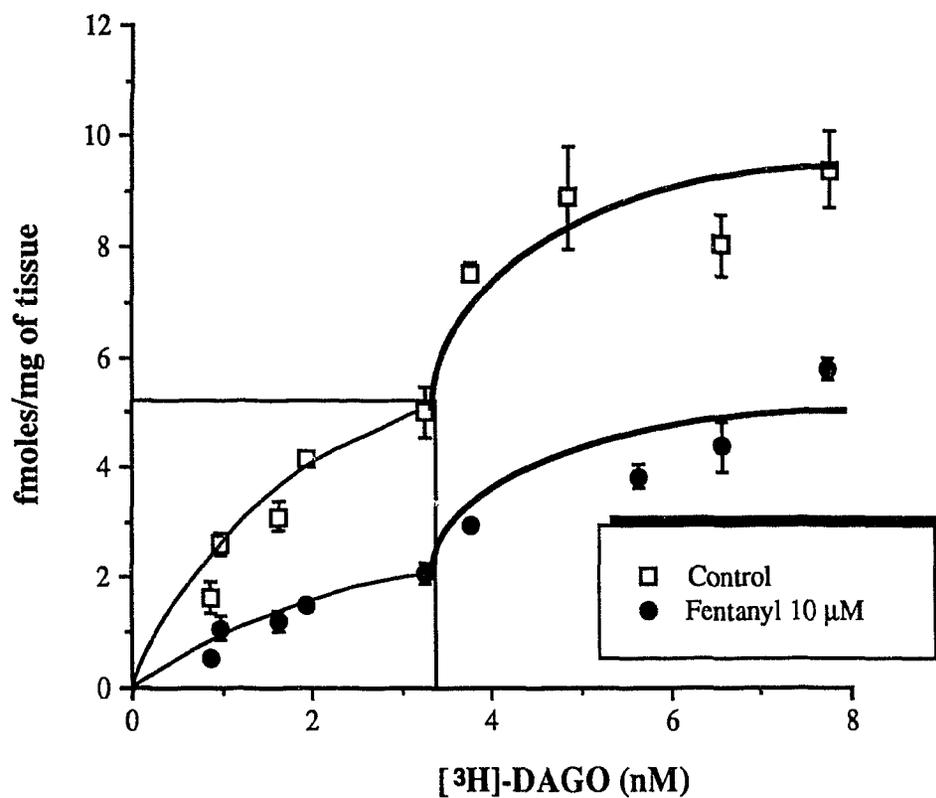
**Fig. 45.** Effect of incubation with different fentanyl citrate concentrations on specific [ $^3$ H]-DAGO (1.5 nM) binding in hypothalamic tissue punches. Incubations were 120 min at 37°C.

### 3a. Quantification of Mu-receptor Down-Regulation with High Concentrations of [<sup>3</sup>H]-DAGO

Using the results obtained above, hypothalamic tissue punches were incubated with fentanyl ( $10^{-5}$ M) or DAGO ( $10^{-5}$ M) for 120 min at  $37^{\circ}\text{C}$  (preceded by 30 min  $37^{\circ}\text{C}$  preincubation) and then assayed with [<sup>3</sup>H]-DAGO (1-8 nM) to obtain  $B_{\text{max}}$  and  $K_{\text{D}}$  values. The DAGO- and fentanyl-induced decreases in [<sup>3</sup>H]-DAGO specific binding experiments are shown in Fig. 46 and Fig. 47 respectively. Note that the curves in both of these figures were not computer-generated but were hand drawn to help illustrate the reasoning behind the range of [<sup>3</sup>H]-DAGO concentrations used in subsequent experiments. Although DAGO and fentanyl both appear to reduce receptor number, Fig. 46 and Fig. 47 indicate that there may be at least two distinct mu-opiate receptor populations possessing different affinities for [<sup>3</sup>H]-DAGO. Mu-receptor densities ( $B_{\text{max}}$ ) examined with a wide range of [<sup>3</sup>H]-DAGO concentrations (1-8 nM) are significantly reduced in agonist-treated tissue (Table 17 and Table 18) whereas receptor affinities ( $K_{\text{D}}$ ) (approximately 4 nM) were unaffected. The shape of the curves within the box (1-3 nM) (Fig. 46 and Fig. 47) suggest that [<sup>3</sup>H]-DAGO binds to another mu-receptor site of higher affinity. Saturation analysis performed on this segment of the curve (Table 17 and Table 18) indicates that the  $B_{\text{max}}$  of the agonist-treated tissue was significantly lower than control whereas the  $K_{\text{D}}$  of the treated group was unchanged. Note that the  $K_{\text{D}}$  values at this end of the curve are less than 50% of those observed when saturation analysis is performed over the entire concentration range. This confirms that high affinity mu-receptors are present in hypothalamic tissue and



**Fig. 46.** Specific [<sup>3</sup>H]-DAGO binding to hypothalamic tissue punches incubated with DAGO (10<sup>-5</sup>M) for 120 min at 37°C. See Table 17 for details.



**Fig. 47.** Specific [<sup>3</sup>H]-DAGO binding to hypothalamic tissue punches incubated with fentanyl citrate ( $10^{-5}$ M) for 120 min at 37°C. See Table 18 for details.

**Table 17.** Effect of DAGO preincubation on specific [<sup>3</sup>H]-DAGO (1-8 nM or 1-3 nM) binding in hypothalamic tissue punches.

<b>Treatment</b>	<b>Kd (nM)</b>	<b>Bmax (fmoles/mg of tissue)</b>
<u>Full Curve (1-8 nM)</u>		
Control	4.6±1.4	11.6±2.1
DAGO (10 μM)	4.7±1.9	5.8±1.5***
<u>Partial Curve (1-3 nM)</u>		
Control	2.1±0.5	6.5±1.5
DAGO (10 μM )	1.9±0.4	2.3±0.6***

Values are mean±s.e.m. \*\*\* p<0.005 when compared to respective control values; Student's t-test.

**Table 18.** Effect of fentanyl citrate preincubation on specific [ $^3\text{H}$ ]-DAGO (1-8 nM or 1-3 nM) binding in hypothalamic tissue punches.

Treatment	Kd (nM)	Bmax (fmoles/mg of tissue)
<u>Full Curve (1-8 nM)</u>		
Control	4.4±1.9	13.6±3.6
Fentanyl 10 $\mu\text{M}$	4.8±1.9	5.1±1.8***
<u>Partial Curve (1-3 nM)</u>		
Control	1.7±0.6	6.5±2.5
Fentanyl 10 $\mu\text{M}$	1.7±0.8	2.1±0.4***

Values are mean±s.e.m. \*\*\*  $p < 0.005$  when compared to respective control values; Student's t-test.

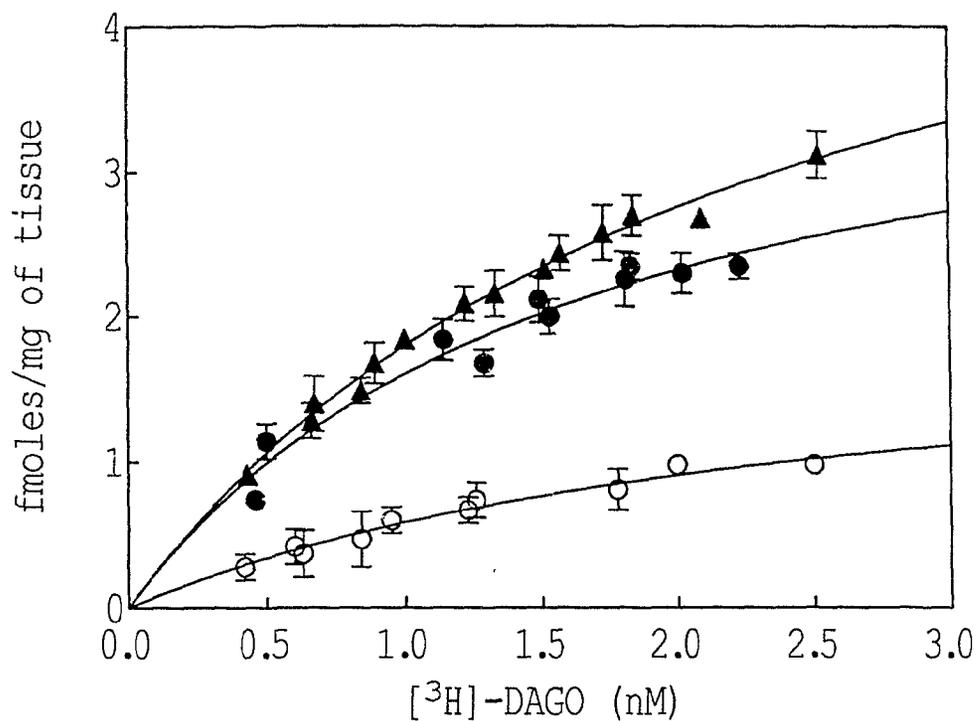
are detectable at low concentrations of [ $^3\text{H}$ ]-DAGO. In order to examine the effect of agonist pretreatment on [ $^3\text{H}$ ]-DAGO binding in a homogeneous population of mu-receptors (i.e. receptors with the same affinity) concentrations of [ $^3\text{H}$ ]-DAGO between 0.5-3 nM were used in all subsequent down-regulation experiments.

### 3b. Quantification of Mu-receptor Down-Regulation with Low Concentrations of [ $^3\text{H}$ ]-DAGO

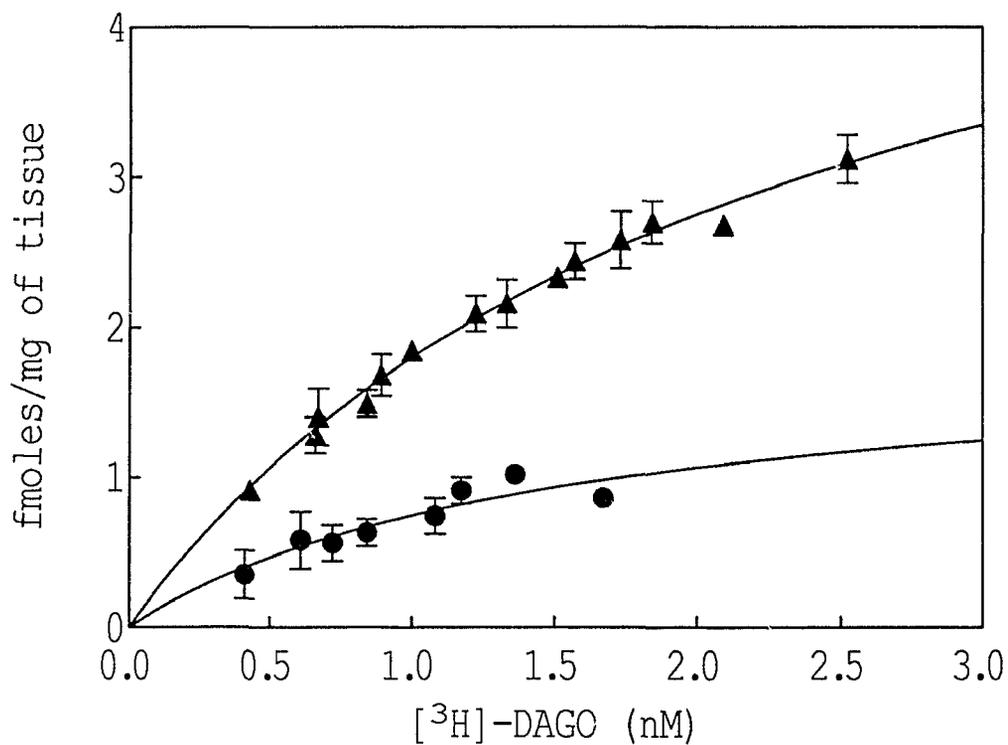
The DAGO-induced ( $10^{-5}$  and  $10^{-6}\text{M}$ ) decrease in specific [ $^3\text{H}$ ]-DAGO receptor binding is shown in Fig. 48. Although both concentrations of DAGO significantly reduced receptor number without effecting  $K_D$  (Table 19), the higher concentration of DAGO ( $10^{-5}\text{M}$ ) was much more effective at decreasing receptor number than the lower concentration ( $10^{-6}\text{M}$ ). Figure 49 demonstrates that when hypothalamic tissue punches were incubated with fentanyl citrate ( $10^{-5}\text{M}$ ) a decrease in specific [ $^3\text{H}$ ]-DAGO is again seen, the  $K_D$  was not significantly different from the control value (Table 19). Similar results were observed when the endogenous opioid  $\beta$ -endorphin ( $10^{-6}\text{M}$ ) was incubated with hypothalamic tissue (Fig. 50). The  $\beta$ -endorphin-induced decrease in  $B_{\text{max}}$  was not associated with a change in receptor affinity (Table 19).

### 4. Effect of Bacitracin on Agonist-induced Mu-receptor Down Regulation

The anti-endocytotic agent bacitracin (Maxfield et al. 1979; Davies et al. 1980) was used to investigate the mechanism of the observed agonist-induced reduction of specific binding (i.e. possible receptor



**Fig. 48.** Effect of DAGO (●)  $10^{-6}\text{M}$ ; (○)  $10^{-5}\text{M}$ ; or (▲) control (without DAGO) on specific  $[^3\text{H}]\text{-DAGO}$  binding in hypothalamic tissue punches. Incubation was 120 min at  $37^\circ\text{C}$ . See Table 19 for details. Curve fitting performed on GraphPAD InPlot

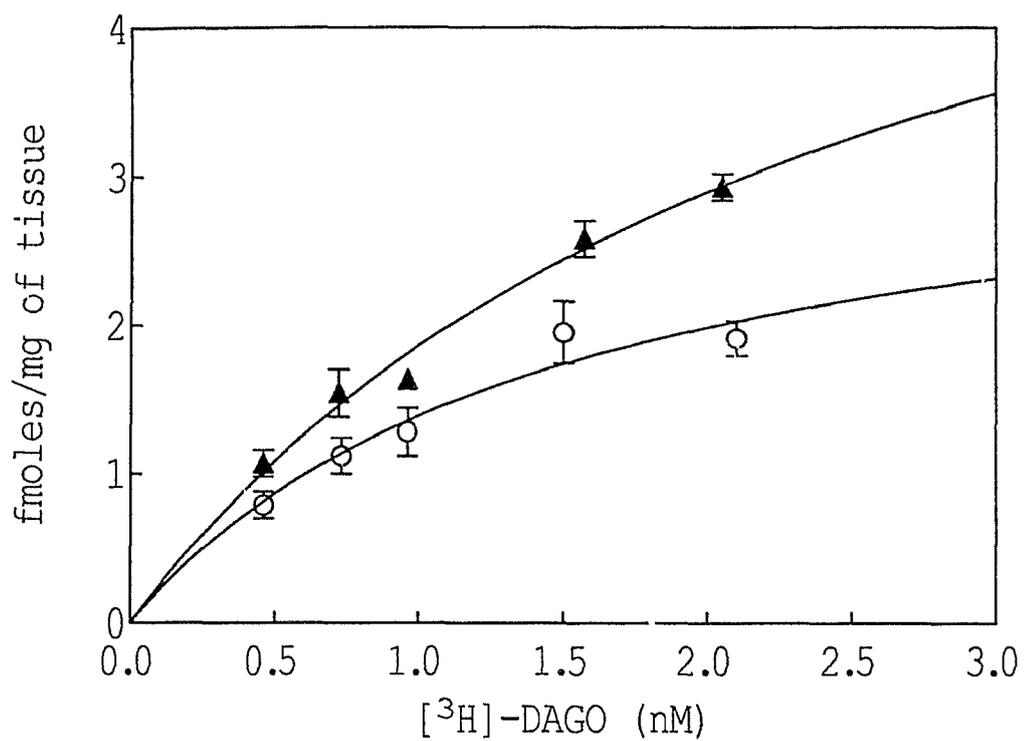


**Fig. 49.** Effect of fentanyl (●)  $10^{-5}$ M; or (▲) control (without fentanyl) on specific [<sup>3</sup>H]-DAGO binding in hypothalamic tissue punches. Incubation was 120 min at 37°C. See Table 19 for details.

**Table 19.** Effect of mu opiate agonist preincubation on specific [<sup>3</sup>H]-DAGO binding (0.5-3 nM) in hypothalamic tissue punches.

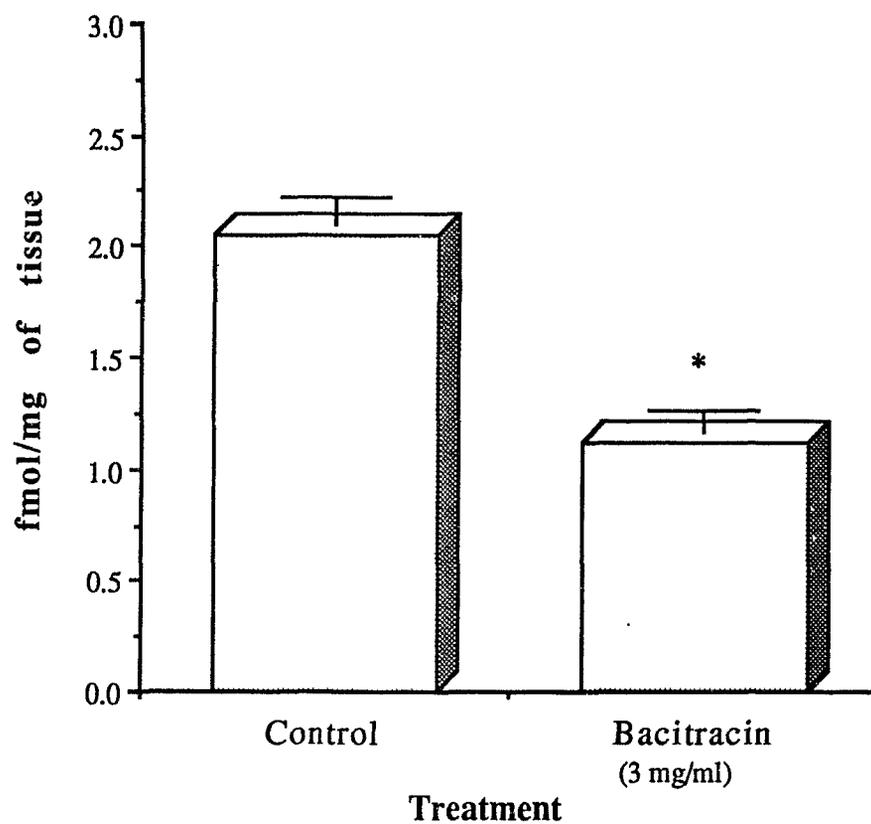
Treatment	Kd (nM)	Bmax (fmoles/mg of tissue)
Control†	2.2±0.2	5.8±0.3
DAGO 1 μM	1.6±0.4	4.1±0.5**
DAGO 10 μM	2.4±0.5	2.0±0.2***
Fentanyl 10 μM	1.5±0.8	1.8±0.5***
β-endorphin - Control	2.5±0.7	6.5±1.2
β-endorphin 1μM	1.5±0.5	3.5±0.5*

\* p<0.05; \*\* p<0.01; \*\*\* p<0.005 when compared with respective control values. †Combined control from 10 experiments. Values are mean±s.e.m.

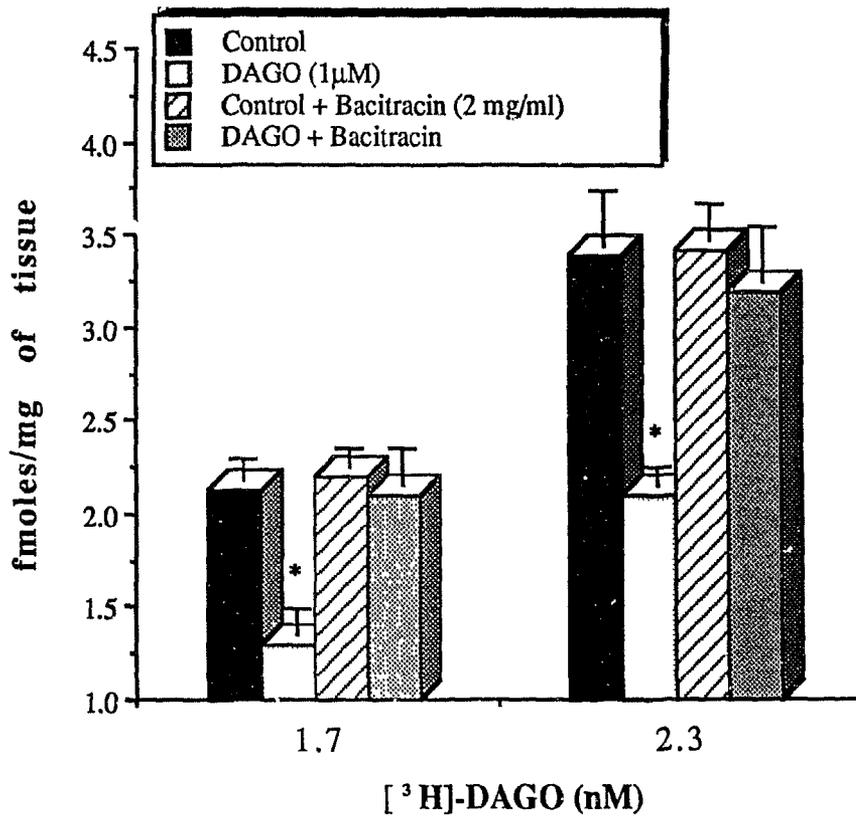


**Fig. 50.** Effect of  $\beta$ -endorphin ( $\circ$ )  $10^{-6}\text{M}$ ; or ( $\blacktriangle$ ) control (without  $\beta$ -endorphin) on specific  $[^3\text{H}]\text{-DAGO}$  binding in hypothalamic tissue punches. Incubation was 120 min at  $37^\circ\text{C}$ . See Table 19 for details.

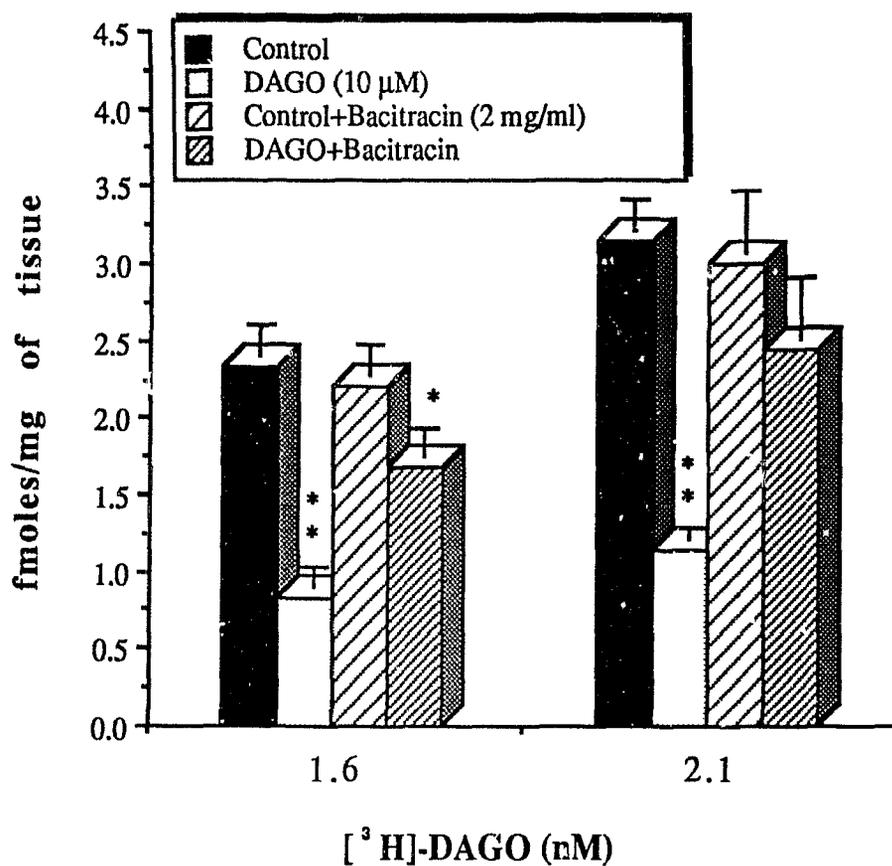
internalization via endocytosis). Preliminary studies (Fig. 51) indicated that when bacitracin was added to the preincubation buffer (DPBS; 37°C for 30 min) concentrations of 0.5-2 mg/ml did not alter control [<sup>3</sup>H]-DAGO binding, whereas a concentration of 3 mg/ml or greater reduced binding by approximately 50%. Therefore, subsequent experiments used a bacitracin concentration of 2 mg/ml. Fig. 52 and Fig. 53 demonstrate the effect of bacitracin preincubation on DAGO-induced down-regulation of mu-opiate receptors. Bacitracin effectively blocked the DAGO (10<sup>-6</sup>M)-induced down-regulation of [<sup>3</sup>H]-DAGO (1.7 nM and 2.3 nM) (Fig. 52; Table 20) without effecting control binding. Conversely, [<sup>3</sup>H]-DAGO binding at 1.7 nM and 2.3 nM in DAGO (10<sup>-6</sup>M)-treated hypothalamic tissue without bacitracin pretreatment, decreased by 39% and 38% respectively. Similar results were observed when the DAGO concentration was increased to 10<sup>-5</sup>M (Fig. 53) although bacitracin did not appear to be as effective at preventing the agonist-induced decrease in specific [<sup>3</sup>H]-DAGO binding (Table 20). Nevertheless, [<sup>3</sup>H]-DAGO binding at concentrations of 1.6 nM and 2.1 nM was only reduced by 24% and 19% respectively in tissue treated with bacitracin and DAGO (10<sup>-5</sup>M). In the absence of bacitracin, specific [<sup>3</sup>H]-DAGO binding, at concentrations of 1.6 nM and 2.1 nM in DAGO-treated tissue was reduced by 64% at both concentrations. In contrast, bacitracin had no effect on the fentanyl-induced reduction of specific [<sup>3</sup>H]-DAGO binding. When hypothalamic punches were incubated with fentanyl citrate (10<sup>-5</sup>M) (Fig. 54), specific [<sup>3</sup>H]-DAGO binding (1.33 nM and 1.63 nM) was significantly reduced in tissue pretreated with and without bacitracin (Table 21).



**Fig. 51.** Effect of preincubation with bacitracin (3 mg/ml of DPBS) for 30 min on specific [ $^3\text{H}$ ]-DAGO binding (1.1 nM) in hypothalamic tissue punches. Control:  $2.0 \pm 0.1$ ; Bacitracin:  $1.1 \pm 0.1$  fmoles/mg of tissue. \* $p < 0.005$ ; Student's t-test. Values are mean  $\pm$  s.e.m.



**Fig. 52.** Effect of preincubation with bacitracin (2 mg/ml) on DAGO (1  $\mu$ M)-induced down regulation of mu opiate receptors quantified with [<sup>3</sup>H]-DAGO (1.7 and 2.3 nM). \* $p < 0.005$  when compared to respective controls; Student's t-test. Values are mean  $\pm$  s.e.m.

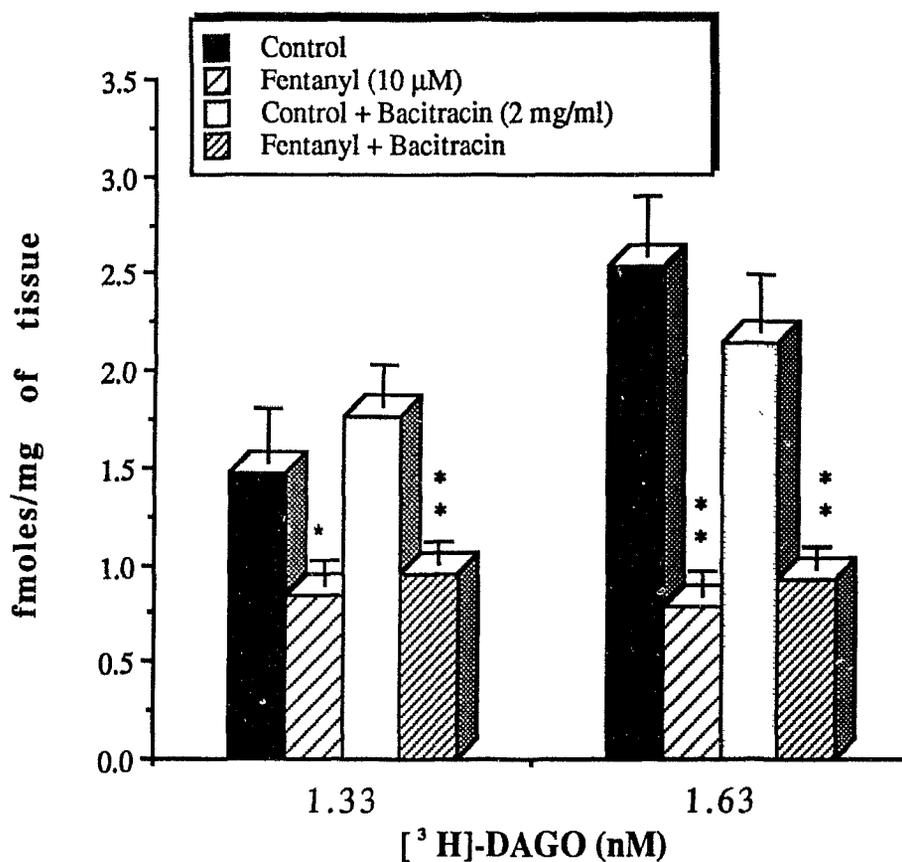


**Fig. 53.** Effect of preincubation with bacitracin (2 mg/ml) on DAGO (10 μM)-induced down-regulation of mu opiate receptors quantified with [<sup>3</sup>H]-DAGO (1.6 and 2.1 nM). \*p<0.05; \*\*p<0.005 when compared to respective controls. Student's t-test. Values are mean±s.e.m.

**Table 20.** Effect of preincubation with bacitracin on DAGO-induced mu-opiate receptor down-regulation in hypothalamic tissue punches assayed at two concentrations of [<sup>3</sup>H]-DAGO

Treatment	[ <sup>3</sup> H-DAGO]	
	1.7 nM (fmoles/mg of tissue)	2.3 nM (fmoles/mg of tissue)
Control	2.1±0.1	3.4±0.3
DAGO 1 µM	1.3±0.1**	2.1±0.1**
Control + Bacitracin (2mg/ml)	2.2±0.1	3.4±0.2
DAGO 1 µM + Bacitracin	2.1±0.2	3.2±0.3
	1.6 nM	2.1 nM
Control	2.3±0.3	3.1±0.2
DAGO 10 µM	0.8±0.1**	1.1±0.1**
Control + Bacitracin (2mg/ml)	2.2±0.2	3.0±0.4
DAGO 1 µM + Bacitracin	1.6±0.1*	2.4±0.1

\* p<0.05; \*\*p<0.005 when compared to respective control values; Student's t-test. Values are mean±s.e.m.



**Fig. 54.** Effect of preincubation with bacitracin (2 mg/ml) on fentanyl citrate (10 μM)-induced down regulation of mu opiate receptors quantified with [<sup>3</sup>H]-DAGO (1.33 and 1.63 nM). \* $p < 0.05$ ; \*\* $p < 0.005$  when compared to respective controls; Student's t-test. Values are mean  $\pm$  s.e.m.

**Table 21.** Effect of bacitracin on fentanyl citrate-induced mu-opiate receptor down-regulation in hypothalamic tissue punches assayed at two concentrations of [<sup>3</sup>H]-DAGO

Treatment	[ <sup>3</sup> H-DAGO]	
	1.33 nM (fmoles/mg of tissue)	1.63 nM (fmoles/mg of tissue)
Control	1.5±0.3	2.5±0.3
Fentanyl 10 μM	0.8±0.1*	0.8±0.1**
Control + Bacitracin (2mg/ml)	1.8±0.2	2.1±0.3
Fentanyl 10 μM + Bacitracin	0.9±0.1**	0.9±0.1**

\* p<0.05; \*\*p<0.005 when compared to respective control values; Student's t-test.  
Values are mean±s.e.m.

## Discussion

Down-regulation of opioid receptors has been extensively studied using a variety of tissue preparations, specifically, the use of neuroblastoma-glioma cell lines in culture containing a homogeneous population of delta-(enkephalin) receptors (Moses and Snell 1983; 1984; Blanchard et al. 1982). The majority of evidence suggests that the down-regulation of opioid receptors is a two-step process (Law et al. 1983; Smith et al. 1988; Blanchard and Chang 1988). The first step involves a receptor-ligand interaction that results in uncoupling of the receptor from its corresponding G protein (i.e. G<sub>i</sub> (inhibitory)) with an accompanying reduction of affinity for agonist binding. This process is rapid and may take only minutes. This process also results in a decrease in adenylate cyclase activity (Rodbell 1980) although chronic opioid treatment results in the gradual loss of opioid inhibition and the return to normal levels of adenylate cyclase activity (Sharina et al. 1975; Smith et al. 1988). Furthermore, when the opioid agonist is removed or an antagonist introduced into the system, adenylate cyclase activity increases above control levels. The second step involves the internalization, or the down-regulation, of the receptor-ligand complex from the cell surface into the interior of the cell. Once internalized, the receptors may be degraded or recycled to the cell surface. Experiments using fluorescent ligands (Hazum et al. 1979) have demonstrated clustering of enkephalin receptors in the N4GT1 cell line. This evidence, together with studies indicating that radioactive agonists accumulate inside these cells (Blanchard et al. 1983), suggests that opioid receptor down-

regulation is accomplished via receptor clustering and subsequent internalization by endocytotic movement. Although numerous studies have tried to integrate the cellular events that occur during down-regulation as possible mechanisms for opiate dependence and tolerance, evidence linking these processes remains limited and inconclusive (Johnson and Fleming 1989).

Numerous studies designed to examine the effect of the opiate agonists on mu-opioid receptors *in vivo* (Klee and Streaty 1974; Perry et al. 1982; Bardo et al. 1982; Lewis et al. 1984; Rothman et al. 1989), have produced conflicting results. The majority of these investigations demonstrated that mu-agonists (i.e. morphine) were ineffective at inducing down-regulation. These studies led to the suggestion that ligand-induced down-regulation may not be a property of mu-receptors (Blanchard and Chang 1988). Many of these *in vivo* experiments used brain homogenization, a process that disrupts cellular integrity, possibly changing receptor conformation and receptor binding properties. *In vitro* studies on mu-receptor regulation, have been confined to a variety of preparations using cell culture techniques (Rogers and El-Fakahany 1986; Puttfarken et al. 1989; Maloteaux et al. 1989). Unfortunately these techniques are often complicated, labour-intensive and require special experimental facilities. The study presented here has taken advantage of a simple, well-described assay (Wilkinson and Wilkinson 1985; Wilkinson et al. 1987; Wilkinson et al. 1990) in which receptors in metabolically active hypothalamic slices/punches are used to study cell-surface regulation in fresh tissue (see Appendix for detailed discussion).

The results of these experiments indicate that the mu-agonists fentanyl citrate, DAGO ([D-Ala<sup>2</sup>-N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol] enkephalin) and the endogenous opioid peptide,  $\beta$ -endorphin, when incubated with hypothalamic tissue punches, are capable of decreasing receptor density ( $B_{max}$ ) without effecting receptor affinity ( $K_D$ ). The down-regulation of mu-receptors with DAGO and fentanyl was dose-dependent and occurred at concentrations which correlated with their relative binding affinities (Wilkinson et al. 1990). The DAGO-induced decrease in receptor number was maximal at 120 min of incubation and corresponds with the exposure time used in the alfentanil-induced down-regulation of mu-receptors in cultured neurons (Maloteaux et al. 1989). The fentanyl-induced decrease in specific [<sup>3</sup>H]-DAGO binding was also maximal after 120 min of ligand exposure, but unlike DAGO, reduced [<sup>3</sup>H]-DAGO binding by 50% after only 30 min of incubation. Similar results using the  $\delta$ -receptor agonist DADLE in the N4TG1 cell line have also been reported (Blanchard et al. 1982; Blanchard et al. 1983; Chang et al. 1982). Using ligand uptake (Blanchard et al. 1983) and down-regulation studies (Blanchard et al. 1982; Chang et al. 1982), these groups demonstrated that receptor number decreases by 60-70% by the first 30 min of agonist exposure. Both uptake and down-regulation is complete by 1 hour post incubation with [<sup>3</sup>H]-DADLE and DADLE respectively.

Initially, the reduction of specific [<sup>3</sup>H]-DAGO binding after agonist incubation, was examined with a wide range of [<sup>3</sup>H]-DAGO concentrations (1-8 nM). The resulting complex saturation curves suggested that two mu-receptors, with differing affinities, were

present within the hypothalamic preparation. Saturation analysis of binding at [<sup>3</sup>H]-DAGO concentrations of 0.5 and 3 nM indicated a site of high affinity with a lower binding capacity when compared to binding analysis of [<sup>3</sup>H]-DAGO at concentrations between 1-8 nM. Note that the  $K_D$  and  $B_{max}$  reported after saturation analysis of the binding at concentrations of [<sup>3</sup>H]-DAGO between 1-8 nM represent the combination of high and low receptor affinities and densities. In subsequent receptor binding studies, only the high affinity site (as determined by saturation analysis), representing a homogeneous population of mu-receptors was used for examining agonist-induced receptor down-regulation. These observations are not unlike those of Moses and Snell (1983; 1984) who measured [<sup>3</sup>H]-Met-enkephalin binding in 108CC15 neuroblastoma x glioma hybrid cells after incubation with DADLE. Scatchard analysis demonstrated that two distinct binding sites, each with its own  $B_{max}$  and  $K_D$ , were present in cells treated with morphine or DADLE.

Competition (Wolozin and Pasternak 1981; Nishimura et al. 1984; Goodman and Pasternak 1985; Zhang and Pasternak 1981) and autoradiographical studies (Moskowitz and Goodman 1985 a,b; Rothman et al. 1987) have provided evidence for the existence of two subclasses of mu-opioid receptors (i.e.  $\mu_1$  and  $\mu_2$ ). The mu-receptor subtype model proposed by Pasternak and Wood (1986) suggests that  $\mu_2$  and delta receptors bind morphine and enkephalin respectively, whereas the  $\mu_1$  receptor subtype binds both morphine and enkephalin with greater affinity than their respective sites. This concept of high- and low-affinity mu-receptor sites supports the observation of two receptors demonstrated here as well

as the observations reported by Moses and Snell (1983; 1984). Whether the two populations described here represent the  $\mu_1$  and  $\mu_2$  sites reported by Pasternak and Wood (1986) is still to be determined.

When fentanyl, DAGO or  $\beta$ -endorphin were incubated with hypothalamic tissue punches, a decrease in  $B_{\max}$  without a change in  $K_D$  was observed. These results suggest that the decrease in specific [ $^3$ H]-DAGO binding was due to reduced receptor number and not residual agonist binding that may have occupied the receptor sites i.e residual agonist would modify the  $K_D$  value. Although studies examining the down-regulation of mu-receptors *in vitro* are limited, these results confirm similar observations of Maloteaux et al. (1989). This group used a variety of tissue preparations and specific mu-agonists from the fentanyl family (i.e. alfentanil, lofentanil, and sufentanil) to demonstrate agonist-induced mu-receptor down-regulation *in vitro*. It is interesting to note that this group did not observe a decrease in receptor number after incubations with morphine, although exposure time and choice of [ $^3$ H] ligand may have been important. Puttfarken et al. (1989) however, using the pituitary 7315C cell line, have shown that morphine is capable of decreasing mu-receptor number in these cells but incubation times between 48 and 72 hours were required.

The ability of the anti-endocytotic agent bacitracin to prevent the DAGO-induced decrease in specific [ $^3$ H]-DAGO binding suggests that the reduction in receptor number may be a result of endocytosis. Bacitracin inhibits the membrane bound enzyme transglutaminase, an important component of the clustering-endocytotic process

(Maxfield et al. 1979; Davies et al. 1980). Previous work with muscarinic acetylcholine receptors in clonal neuroblastoma-glioma hybrid cells has also demonstrated the anti-endocytotic ability of bacitracin (Ray et al. 1989). The observation that bacitracin was unable to prevent the fentanyl-induced down-regulation suggests that residual fentanyl occupies the receptors and thus may be responsible for the reduced [<sup>3</sup>H]-DAGO binding. However, saturation binding analysis indicates that the affinity of the down-regulated receptor was not significantly different from control values, which would argue against a fentanyl bound receptor. One possible explanation is that fentanyl, like morphine is an alkaloid, whereas DAGO is a peptide. These ligands may therefore down-regulate mu-receptors by different mechanisms. The differences between alkaloids and peptides have been demonstrated in neuroblastoma-glioma hybrid cells although the majority of these studies suggest that alkaloids are unable to down-regulate opioid receptors (Chang et al. 1982; Law et al. 1983; Zukin and Tempel 1986; Smith et al. 1988).

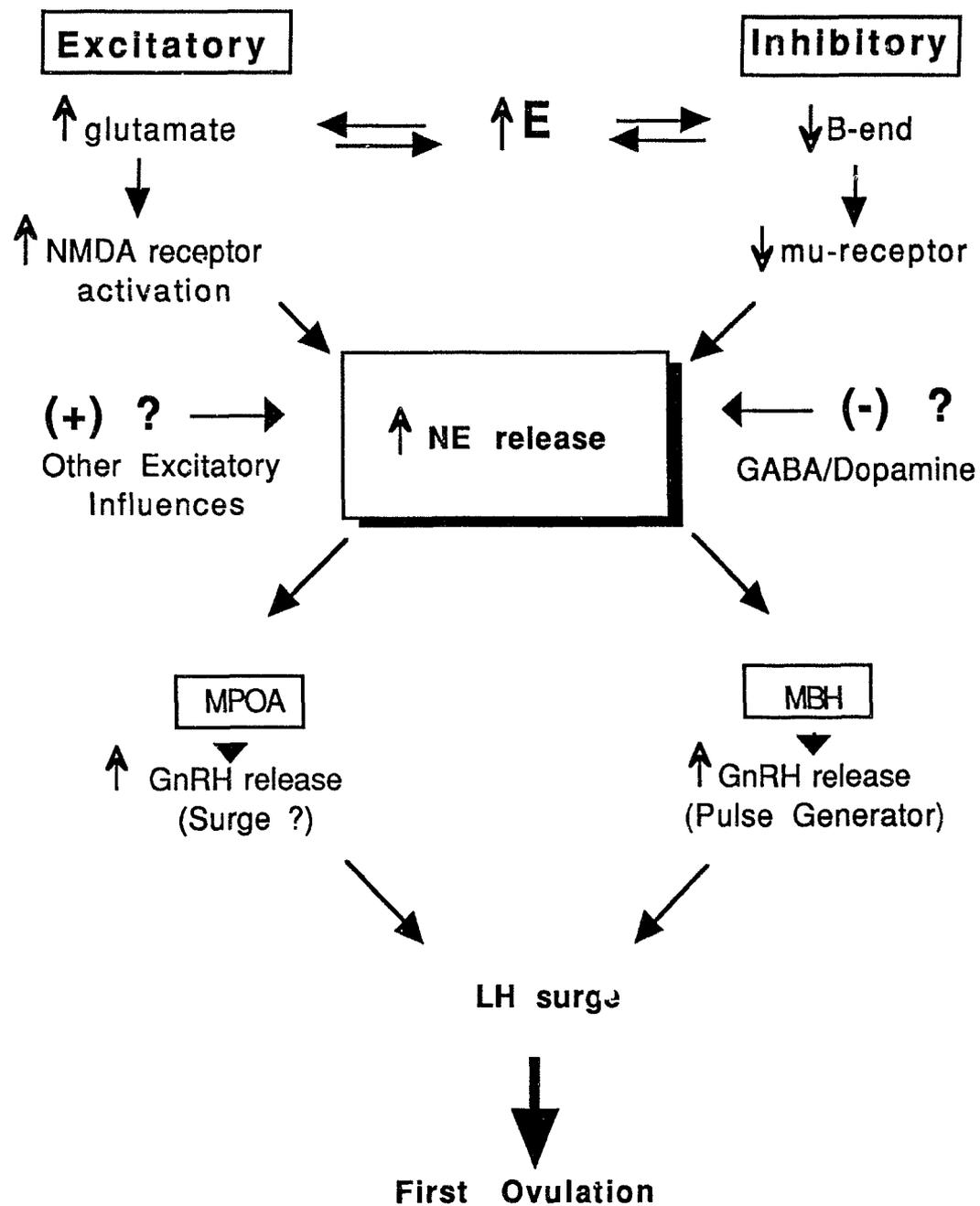
In conclusion, the data suggest that mu-receptor down-regulation can be detected in the *in vitro* system described. Important future experiments would be to test whether morphine is also capable of inducing a reduction in mu-specific binding and to examine [<sup>3</sup>H]-DAGO binding in hypothalamic punches obtained from the morphine-treated rats described in this section (see Sect. III Results p. 155). Finally, the question of a possible reversal of the down-regulation could also be investigated. It is not possible at present to identify the cells which contain the mu-receptors although the work of Maloteaux et al. (1989) implies that cultured neurons certainly are one source.

## Summary

The precise mechanism that mediates the increase in GnRH secretion and subsequent LH surge during first ovulation still remains elusive. Studies presented here, however, indicate that distinct excitatory and inhibitory pathways may play an integral role in the neural control of puberty in the female rat. Figure 55 represents a synthesis of two possible pathways. On the left side of the diagram, the excitatory pathway that may mediate GnRH release is illustrated. NMDA receptor activation by glutamate results in an increase in NE release at the GnRH nerve terminal and possibly the GnRH cell bodies in the MPOA as well. The increase in GnRH secretion produces an LH surge. Elevated estrogen levels, during the peripubertal stage, could also potentiate NMDA receptor activation (Smith 1989) and amplify GnRH release, and subsequent LH surge. NMDA receptor activation in the MBH may increase GnRH release and regulate GnRH pulsatility either directly or via NE release.

The inhibitory pathway, mediated by the endogenous opioid  $\beta$ -endorphin, is illustrated on the right side of the diagram. An increase in estrogen during the peripubertal period may decrease  $\beta$ -endorphin content in the MBH or MPOA resulting in the removal of an 'opioid brake'. With the removal of opioid inhibition NE secretion increases and results in higher GnRH and LH secretion. It is also possible that opioid tone may decrease as a result of the down-regulation of mu-opioid receptor number.

Experiments presented in this thesis have demonstrated that both pathways play a significant role in mediating the neural control of



**Fig. 55.** Schematic summary of possible neural mechanisms that mediate the LH surge observed on the day of first ovulation. Abbreviations. N-methyl-D-aspartate (NMDA); estrogen (E);  $\beta$ -endorphin (B-end); neopinephrine (NE); medial preoptic area (MPOA); mediobasal hypothalamus (MBH); gonadotropin-releasing hormone (GnRH); luteinizing hormone (LH).

sexual maturation. Note, however, that these are not the only neuromodulators that regulate the onset of puberty. Afternoon activation of non-NMDA receptors during sexual maturation may also be important for generating the LH surge required for first ovulation. The removal of other inhibitory influences such as GABA or dopamine cannot be excluded from the list of neurotransmitters that mediate GnRH release. The precise site mediating GnRH release has not yet been determined although activation at both the MPOA (site of GnRH cell bodies) and the MBH (site of GnRH terminals) appear to be important.

## Conclusions

- 1.) Hypothalamic glutamate receptors of the NMDA-subtype comprise a significant regulatory component of the neural control of sexual maturation. The delay in V.O as a result of the block of NMDA receptors with antagonists is dependent on age and timing of the injections.
- 2.) Single daily injections with NMDA not only result in an advanced day of V.O. but also synchronize the number of rats experiencing V.O. on a given day.
- 3.) NMDA-induced LH secretion in female rats is greatest during the juvenile period and decreases as sexual maturation approaches.
- 4.) Studies presented here have demonstrated that the excitatory effect of NMDA on LH secretion is mediated through norepinephrine release, although the involvement of other neurotransmitters and/or receptors remains largely unexplored.
- 5.) Neonatal MSG treatment appears to remove an inhibitory hypothalamic restraint mechanism and culminates in precocious first ovulation. The MSG-induced elimination of opioid neurons in the arcuate nucleus may be one essential component of this restraint. However, MSG treatment does not affect the mechanism that mediates the NMDA-induced release of LH. The precociousness observed in MSG-treated animals varies with the dose and injection

schedule employed. MSG treatment later than postnatal day 10 has no effect on the timing of V.O.

6.) The use of c-fos immunostaining has demonstrated for the first time that NMDA-induced cellular activity is localized within the arcuate nucleus and the subfornical organ, two areas that also mediate GnRH/LH release. The appearance of c-fos immunoreactivity can be prevented by NMDA antagonists and is also significantly decreased in adult male rats when compared to prepubertal male rats. This reduction parallels that of NMDA-induced LH release. In contrast, the greatly reduced c-fos immunostaining in MSG-treated animals is clearly not incompatible with a normal NMDA-induced increase in LH. This suggests that there may be a discrete group of neurons responsible for GnRH secretion, within the arcuate nucleus, that is unaffected by neonatal MSG treatment

7.) Opioids play an important role in the neural regulation of sexual maturation. Continuous or discontinuous exogenous opiate treatment are both capable of delaying but not preventing V.O. An excitatory neural drive, possibly glutamate-mediated, could negate opiate inhibition and permit first ovulation to occur. Indeed, the observation that MK-801 treatment can delay V.O. in fentanyl-treated rats suggests that opioidergic and glutamatergic control of GnRH/LH release is mediated through concurrent but distinct pathways. However, since V.O. was not prevented, this implies that another excitatory influence, such as non-NMDA receptor activation,

may be an additional important component of the neural drive that culminates in the first LH surge and ovulation.

8.) A receptor binding assay using fresh hypothalamic tissue punches has been developed and used to successfully demonstrate the down-regulation of mu-opiate receptors. Both opioid peptides and alkaloids induce a decrease in receptor number without affecting binding affinity, although the actual mechanism of down-regulation induced by each compound may not be the same. This assay provides an effective technique with which to examine opioid receptor regulation in rats treated with opiates.

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