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NMDA RECEPTORS AND THE NEURAL CONTROL OF PUBERTY IN FEMALE RATS

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This thesis is dedicated to my parents, Arlene Mae Smyth and
Dennis Lyall Smyth.

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ABSTRACT

N-methyl-D-aspartic acid (NMDA) subtype receptors are important in regulating sexual maturation. Daily treatment with NMDA (20 mg/kg; s.c.) from 24 days of age advances first ovulation in immature female rats while daily injections of gonadotropin releasing hormone (GnRH; 5 ng/100 g) do not. This suggests that NMDA alters hypothalamic control, not directly related to LH secretion. There is a critical period for effectiveness as daily injections from day 16 to 20 or immediately preceding spontaneous first ovulation do not induce precocious puberty. An LH dose-response curve for NMDA (P28) demonstrates that in rats previously treated with NMDA (P24 to P27), the LH response is lower than in rats previously treated with saline (P24 to P27). The site of action of NMDA was localized by c-fos immunoreactivity (FLI) which was identified in a dose-dependent manner in circumventricular organs (CVOs) following treatment with NMDA or monosodium glutamate (MSG). In this second model of precocious puberty, the arcuate nucleus (ARCN) of MSG-treated neonatal rats showed a dose- and age-dependent increase in FLI. NMDA- but not MSG-induced FLI in neonates was blocked by MK-801 even though MK-801 has been shown to prevent MSG-induced early puberty.

Precocious maturation may be mediated through focal lesions in the ARCN. The stress protein, HSP72, increased in an NMDA dose-dependent manner in the ARCN of female rats. HSP72-like immunoreactivity (-li) peaked between 12 and 18 hrs. and disappeared 7 days post-treatment and was not evident in neonatal rats (P2 to 10) at any time following MSG- or NMDA-treatment.

Growth factors are also believed to regulate puberty. High levels of bFGF mRNA were localized by reverse transcription polymerase chain reaction (RT-PCR) in rat hypothalamus and were found to decrease 40% at VO (P29) in NMDA-treated rats. Immunocytochemistry showed more bFGF-positive cells in the ARCN in NMDA-treated rats on P29; these fell significantly by P33.

The data has shown that there is a critical time point where daily subcutaneous treatment with NMDA accelerates sexual maturation in the female rat. NMDA is acting on brain cells in the ARCN and other CVOs and may induce "stress" or injury in the ARCN. In addition, bFGF may be involved in the process leading to NMDA-induced precocious puberty.

LIST OF ABBREVIATIONS

-li	- like immunoreactivity
1°	primary (antibody)
2°	secondary (antibody)
2-DG	2-deoxyglucose
ACPD	trans-1-aminocyclopentane-1,3-dicarboxylate
ACTH	adrenocorticotropin hormone
aFGF	acidic fibroblast growth factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPOA	anterior medial preoptic area
AP	area postrema
AP-1	activator protein-1 (binding site)
AP-3	($\pm$)-2-amino-3-phosphonopropionic acid
AP-4	($\pm$)-2-amino-4-phosphonobutyric acid
AP-5 or APV	($\pm$)-2-amino-5-phosphopentanoic acid
AP-7	($\pm$)-2-amino-7-phosphoheptanoic acid
AQ	animal quarters
ARCN	arcuate nucleus
ASP	aspartate
AT	area tempestas
ATP	adenosine triphosphate
BBB	blood brain barrier
BCA	bilateral carotid artery (occlusion)
BDNF	brain-derived neurotrophic factor

bFGF	basic fibroblast growth factor
bFGF.R	basic fibroblast growth factor receptor
BiP	immunoglobulin binding protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CGS 19755	cis-4-(phosphonomethyl)piperidine-2-carboxylic acid
CL	corpora lutea
CNQX	6-cyano-7-nitroquioxaline-2,3-dione
CNS	central nervous system
CPP	(±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
CRB	cerebellum
CRF	corticotrophin-releasing factor
CTX	cortex
CVO	circumventricular organs
DA	domoic acid
DMN	dorsomedial (arcuate nucleus)
DNA	deoxyribonucleic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
dNTP	deoxynucleotide triphosphates
DPBS	Dulbecco's phosphate buffered saline
DTT	dithiothreitol
E(number)	embryonic day
E_2	estradiol
EAA	excitatory amino acid
EB	estradiol benzoate

EC	entorhinal cortex
EEG	electroencephalogram
EGF	epidermal growth factor
EM	electron microscopy
ER	endoplasmic reticulum
ERE	estrogen response element
FSH	follicle stimulating hormone
FLI	fos-like immunoreactivity
Fra	Fos related antigens
GABA	γ-aminobutyric acid
GH	growth hormone
Glu	glutamate
GluR1 to R7	glutamate receptor subunits (1 to 7)
Gly	glycine
GRP 78	glucose-regulated peptide 78
HBGF	heparin binding growth factor
HMW	high molecular weight
hr	hour
HCA	homocysteate
H-P-G	hypothalamic-pituitary-gonadal (axis)
HSC	heat shock cognate protein
HSP	heat shock protein
HSP72-li	heat shock protein 72 - like immunoreactivity
IEG	immediate early gene
IGF-I or II	insulin-like growth factor I or II
i.v.	intravenous
i.p.	intraperitoneal

IP3	inositol triphosphate
ISHH	in situ hybridization histochemistry
IWK	Izaak Walton Killam Hospital
KA	kainic acid
kDa	kiloDaltons
LDH	lactate dehydrogenase
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LMW	low molecular weight
MAP	microtubule associated protein
MBH	mediobasal hypothalamus
MCA	middle cerebral artery (occlusion)
ME	median eminence
mGLUR	metabotropic glutamate receptor
min	minute
μM	micromolar
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo-[A,D]-cyclohepten-5,10-imine-maleate
mM	millimolar
mRNA	messenger RNA
M-M.V	Moloney-Murine Leukemia Virus
MS	medial septum
MSG	monosodium glutamate
NA	noradrenergic
NBQX	6-Nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione
NE	norepinephrine
NGF	nerve growth factor

NGF.R	nerve growth factor receptor
NHS	normal horse serum
NMA	N-methyl-D,L-aspartic acid
NMDA	N-methyl-D-aspartic acid
NMDA.R	N-methyl-D-aspartic acid receptor
NPY	neuropeptide Y
NR1, NR2	NMDA receptor subunits
NRS	normal rabbit serum
NSB	nonspecific binding
OVLT	organum vasculosum of the lamina terminalis
OVX	ovariectomized
P(number)	postnatal day
PB	phosphate buffer
PBS	phosphate buffered saline
PCC	posterior cingulate cortex
PCP	phencyclidine
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
pM	picomolar
PMSG	pregnant mares' serum gonadotrophin
POA	preoptic area
PRC	photoreceptor cells
PRL	prolactin
ProE	proestrus
PVN	paraventricular hypothalamic nucleus
PTZ	pentylenetetrazol

QA	quinolinic acid
RAI	rapid acceleration injury
RC	retrosplenial cortex
RGC	retinal ganglion cells
RNA	ribonucleic acid
RT	reverse transcription
s.c.	subcutaneous
SAL	saline
SAP	sympathoadrenal preganglionic (neurons)
SB	specific binding
SCN	suprachiasmatic nucleus
SCO	subcommissural organ
SCP	sodium chloride phosphate (buffer)
SD	Sprague-Dawley
SFO	subfornical organ
SON	supraoptic nucleus
SSC	standard saline citrate
tau	microtubule associated protein species
Tau	taurine
TC	total counts
TCP-1	tailless protein complex-1
TGFα	transforming growth factor α
TRiC	TCP-1 ring complex
TX	Triton x-100
Ub	ubiquitin
vDB	vertical limb of diagonal band of Broca
VL	ventrolateral (arcuate nucleus)

VM	ventromedial (arcuate nucleus)
VMH	ventromedial hypothalamus
VMN	ventromedial hypothalamic nucleus
VO	vaginal opening
wt	weight

ACKNOWLEDGEMENTS

CHAPTER 1: THE ROLE OF GLUTAMATE IN THE NEUROENDOCRINE SYSTEM

I. Definition of Puberty

A critical event in each of our lives is the attainment of sexual maturity. Webster's dictionary defines puberty as "the period of becoming first capable of reproducing sexually, marked by maturation of the genital organs, development of secondary sex characteristics, and, in the human and in higher primates, by the first occurrence of menstruation in the female". The physiological ability to reproduce and the physical changes associated with puberty are brought about gradually through gonadal activation. The low levels of gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which are characteristic of childhood rise gradually through puberty, and in the female, trigger an increase in the ovarian production of estrogens. Two manifestations of puberty are the sleep-associated increases in basal LH levels, and amplitude of LH pulses. These endocrinological changes are correlated with the onset of puberty in boys and girls and are driven by a central mechanism: the so-called "luteinizing hormone-releasing hormone (LHRH) pulse generator" of the hypothalamus. The onset of puberty appears to be regulated by central (brain) as opposed to peripheral (ovary) processes. The observation that the experimental administration of pulses of LHRH to juvenile rats and monkeys induces precocious first ovulation has led to the firmly held view that there is a neuronal process which leads to first ovulation. Indeed, the fact that the prepubertal appearance of a diurnal pattern of pulsatile LH secretion occurs even in the absence of gonads in rats, primates and children stresses the point that the initiation of puberty is a brain-driven, gonad-independent event.

II. The Rat as a Model

The rat is both a convenient and appropriate animal model to study reproductive development (Ojeda and Urbanski, 1994). In the female rat, outward signs of sexual maturation are readily detectable. For example, vaginal opening (VO) usually occurs on the day of first ovulation. Also, there are many similarities in the basic mechanisms underlying female reproductive function in rats and humans, including: control of LHRH release by neurotransmitters, steroid positive and negative feedback, and hormonal control of ovarian follicular development (Ojeda and Urbanski, 1994). A striking similarity between rat and human development is the prepubertal appearance of a diurnal pattern of pulsatile LH secretion. In the female rat for example, basal LH levels rise and the amplitude of the LH pulses increases until ovulatory surges of LH appear in the afternoon. Boyar (1978) and Watanabe and Terasawa (1989) believe that the rat is a suitable model for the study of these prepubertal diurnal changes in LH secretion in primates and in children. Due to the hormonal similarity of rat and human sexual development, many of the findings from rat experiments, including those in this thesis, may be extrapolated to the onset of puberty in humans.

III. Mechanisms of the Onset of Puberty

A precise description of the neural mechanism(s) that underlies sexual maturation remains elusive (Ojeda and Urbanski, 1994; Ojeda, 1991). However, several theories have been proposed to account for the onset of puberty.

Gonadostat Hypothesis

One of the earliest explanations of the onset of puberty is termed the "gonadostat resetting hypothesis" (Hohlweg and Junkmann, 1932; Ramirez and McCann, 1963 and 1965). This hypothesis states that as a rat approaches puberty there is a decrease in the sensitivity of the hypothalamic-pituitary system to steroid negative feedback. The gradual removal of steroid inhibition would allow gonadotrophin levels and, in turn, steroid levels to rise. Ramirez and McCann (1963, 1965) showed that castrated immature male and female rats were more sensitive to the steroid negative feedback effects on pituitary LH secretion than adults. Steele and Weisz (1974) used radioimmunoassay technology to show that estradiol treatment of prepubertal ovariectomized female rats completely suppressed LH levels. However, they noted that there was an abrupt rise in LH or an "escape" from negative feedback at approximately the day of VO. In addition, hypothalamic implants of estradiol were more effective at inhibiting the post-castration rise in gonadotrophins in the juvenile compared to the adult (Docke et al., 1978).

That immature rats are more sensitive to the inhibitory effects of estradiol than adults is well accepted and has been confirmed in both male (Smith et al., 1977; Negro-Vilar et al., 1973) and female (Steele and Weisz, 1974; Eldridge et al., 1977) rats. Work from Ojeda's laboratory, however, has shown that the resetting of the gonadostat may occur after first ovulation. For instance, Andrews et al. (1981) demonstrated that a relatively low concentration of estradiol was needed to suppress LH secretion as late in puberty as the morning before first ovulation. This group showed that the strong inhibitory effects of estradiol were promptly lost on first estrous. Therefore, it appears that the resetting of the gonadostat may be a result rather than a cause of the onset of puberty. More plausible explanations of sexual maturation may exist in a neuronal process activating

pulsatile LHRH release that is independent of gonadal input (Ojeda and Urbanski, 1987).

Removal of a Hypothalamic Restraint

The hypothesis that puberty occurs coincident with the removal of a hypothalamic restraint arose from the research of Donovan and van der Werff ten Bosch (1965). They showed that an electrolytic lesion of the hypothalamus resulted in precocious puberty and hypothesized that the lesion effectively eliminated a neural substrate exerting inhibitory control over sexual development. These findings have been confirmed many times (Bogdanove and Schoen, 1959; Gellert and Ganong, 1960; Schiavi, 1964; Advis and Ramirez, 1977) and the evidence has shown that lesioned animals reach true precocious puberty because they have normal post-pubertal reproductive functions (Advis and Ramirez, 1977; Bar-Sela, 1965). However, the stimulatory effects of the lesion are not specific. For instance, lesion-induced early puberty is found when lesions are placed in many areas of the hypothalamus or when placed in animals of different ages (Schiavi, 1964; Advis and Ramirez, 1977; Ruf et al., 19⁻ᵌ\.

There also appears to be substantial evidence for an inhibitory neurochemical mechanism controlling sexual development. Endogenous opioid peptides exert a tonic inhibitory control over LHRH secretion (for review see Wilkinson and Landymore, 1989). Bhanot and Wilkinson (1983) reported that the opioid inhibition is gradually removed in parallel with the decrease in steroidal sensitivity as sexual maturation proceeds. The authors suggested that the opioids may be a neurochemical component of the resetting of the gonadostat.

Increase in Excitatory Input to the Hypothalamus

The lesion-induced advancement of puberty may not be due exclusively to the removal of inhibitory inputs but may also be attributed to the activation of the hypothalamic-pituitary-gonadal axis. It has been reported (Ruf et al., 1975; Young-Lai et al., 1976; Moll et al., 1976) that unilateral electrolytic lesions of the hypothalamus increase circulating levels of LH and estrogen within 1 hour of treatment and increase synaptogenesis on the contralateral side of the lesion (Ruf, 1982). In addition, lesions of the hypothalamus appear to advance the removal of steroid negative feedback (Ruf et al., 1975).

A pubertal increase in excitatory input to the hypothalamus is also known as the synaptogenic hypothesis of puberty. It is based on the premise that the anatomical and functional integration of LHRH neurons with their associated neuronal circuitry is necessary to establish synchronized, pulsatile release of LHRH (Ojeda et al., 1989). Alone or in combination with the removal of a restraint, puberty may occur coincident with an increase in excitatory neural input. LHRH neurons receive a variety of synaptic inputs from, for example, noradrenergic (NA), opioid, γ-aminobutyric acid (GABA)-ergic, and dopaminergic (DA) neurons. LHRH neurons are also reported to synapse with each other (Leranth et al., 1985). Therefore, "peripubertal changes in hypothalamic connectivity might play a role in the activation of the reproductive system" (Ruf, 1982).

In accordance with this hypothesis is the observation that LHRH neurons change their morphology during development (Wray and Hoffman, 1986). In male and female rats, the number of smooth LHRH cells decreases and the number of LHRH cells with spine-like processes (irregular) increases during development and stabilizes shortly after sexual maturation (Wray and Hoffman, 1986). The affinity of ingrowing axon terminals for LHRH neurons may be

influenced by the development of the spine-like structures and may play an integral role in the organization of neuronal circuits which determine the pattern of LHRH secretion (Wray and Hoffman, 1986).

Matsumoto and Arai (1976) were the first to attempt to correlate the formation of axodendritic synapses in the arcuate nucleus of the hypothalamus with the attainment of puberty. They found that the rising number of axodendritic synapses and overall maturity of the neurons in the arcuate nucleus (ARCN) plateaus at approximately the time of first ovulation in female rats. Similar results were obtained by Anderson (1982) who showed that the anterior superior and posterior superior portions of the preoptic area (POA) showed a marked increase in dendritic spine density at the age of VO. This association between puberty and neuronal development is also found in pigs in which an oxytocin- and vasopressin-containing nucleus in the hypothalamus significantly increases in size by increasing neuron number around the time of puberty (van Eerdenburg et al., 1990). Meyer et al. (1978) have also shown that there is a peak in spine density in CA1 pyramidal neurons of the hippocampus which coincides with puberty in male mice. Neuronal development can readily be observed when sexual maturation is chemically accelerated. Stimulation of precocious puberty in rats by pregnant mare serum gonadotropin (PMSG; Matsumoto and Arai, 1977) or by estradiol benzoate (EB; Clough and Rodriguez-Sierra 1983) also significantly increases the number of synapses in the ARCN. Thus, the association between puberty and degree of neuronal connectivity is observed in normal puberty and in various models of precocious puberty. This strongly suggests that "the functional maturation of the onset mechanism of puberty is correlated with the growth rate of the neuronal components of the hypothalamus and POA and also with the establishment of the neural connections with the converging afferent systems." (Matsumoto and Arai, 1977). However, little is

known concerning the type of synapses formed (excitatory or inhibitory) or the nature of the neurotransmitters (see Wilkinson et al., 1979).

There are several excitatory neurotransmitter systems which have been implicated in the regulation of sexual maturation: noradrenergic, serotoninergic and glutamatergic (Brown, 1971; Aguilar et al., 1990; Sarkar et al., 1981; Ojeda and Urbanski, 1987). Previous work in this laboratory and others has firmly established the importance of the excitatory neurotransmitter, glutamate, in the hypothalamic regulation of the onset of puberty (Ojeda and Urbanski, 1987; MacDonald and Wilkinson, 1990, 1992). The central theme of this thesis is the involvement of glutamate and, specifically, its N-methyl-D-aspartate (NMDA) subtype receptor in the central process controlling sexual maturation of the female rat. Therefore, the remaining portion of the introduction is devoted to a description of glutamate receptors, the synaptogenic effects regulated by the NMDA receptor and its neuroendocrine role in anterior pituitary hormone secretion and sexual maturation.

IV. The Neurotransmitter Glutamate

The Glutamate Receptors

Until quite recently, the role of glutamate in the central nervous system (CNS) has been controversial but it has now been established as the most abundant excitatory amino acid in the brain and as the major neurotransmitter in the hypothalamus. Van den Pol et al. (1990) reported that in rats, all cells in the paraventricular and arcuate nuclei receive synaptic contacts from glutamate-positive axons. As a neurotransmitter, glutamate is released from synaptic boutons in a Ca^{2+}-dependent manner. It binds to specific postsynaptic receptor subtypes and excites the postsynaptic neuron (see Table 1). Glutamate plays a role in many physiological mechanisms ranging from processing of information to synaptic plasticity and neurotoxicity. Glutamate also appears to play a role in such physiological phenomena as learning and memory and in neurodegenerative diseases such as schizophrenia and Alzheimer's (for review see McDonald and Johnston, 1990a).

The functional diversity of this neurotransmitter can largely be explained by the number of disparate glutamate receptors. The different subtypes of the glutamate receptors are named after their selective agonists and form two classes: the ionotropic and the metabotropic receptors. The ionotropic receptors contain an integral ligand-gated ion channel and include the NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and the kainic acid (KA) receptor. The metabotropic receptors are coupled to G proteins and include the trans-1-aminocyclopentane-1,3-dicarboxylate (ACPD) receptor and the L-2-amino-4-phosphonobutyric acid (AP-4) receptor (Monaghan et al., 1988).

Excitatory Amino Acid Receptor Subtypes
and their Agonists and Antagonists

| Class | Receptor Subtypes | Pharmacologic characteristics | |
		Agonist	Antagonist
NMDA	NR1	NMDA/NMA	MK-801
	NR2A	Aspartate	Mg2+
	NR2B	L-Glutamate	AP-5
	NR2C	Quinolinate	AP-7
	NR2D	Ibotenate	PCP
			CPP
			ketamine
AMPA	GluR1	AMPA	CNQX
	GluR2	Quisqualate	DNQX
	GluR3	Kainate	NBQX
	GluR4	L-Glutamate	
Kainate	GluR5	Kainate	CNQX
	GluR6	Domoate	DNQX
	GluR7	L-Glutamate	Kynurenate
	KA-1		
	KA-2		
AP-4	mGluR6	L-AP-4	
	mGluR7		
ACPD	mGluR1	t-ACPD	
	mGluR2		
	mGluR3		
	mGluR4		
	mGluR5		

Table 1. Classification of EAA receptors in the mammalian CNS.
Abbreviations are as follows: NMDA: N-methyl-D,L-aspartate; NMA: N-methyl-D,L-aspartic acid; AP-4: L-2-amino-4-phosphonobutyric acid; AP-5 or APV: DL-2-amino-5-phosphonovaleric acid (competitive NMDA antagonist); AP-7: 2-amino-7-phosphonoheptanoic acid (competitive NMDA antagonist); CNQX, DNQX and NBQX or quinoxaline derivatives (competitive non-NMDA antagonists); MK-801 (noncompetitive NMDA antagonist); CPP: (±)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; PCP: phencyclidine; ACPD: trans-1-aminocyclopentane-1,3-dicarboxylate; AP-4: (±)-2-amino-4-phosphonobutyric acid. Modified from Brann and Mahesh (1992).

The NMDA receptor is made up of at least five pharmacologically distinct binding sites (Monaghan et al., 1988). These include a glutamate-binding site, a glycine-binding allosteric regulatory site, a voltage-dependent Mg^{2+} binding site, a Zn^{2+} binding site and a site in the ion channel for channel blockers such as phencyclidine (PCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo-[A,D]-cyclohepten-5,10-imine-maleate (MK-801) (see Figure 1).

Cloning of the Glutamate Receptors

Since the cloning of the first glutamate receptor subunit (GluR1; Hollmann et al., 1989) there has been an explosion of scientific papers identifying several members of the KA, AMPA and metabotropic glutamate receptor families. The various subunits of the ionotropic non-NMDA glutamate receptors have been divided into 3 classes. The first class is made up of GluR1 to R4 and are known as AMPA-selective glutamate receptors (Boulter et al., 1990; Keinanen et al., 1990 and Nakanishi et al., 1990). The second class has three members and is comprised of GluR5 (Bettler et al., 1990), R6 (Egebjerg et al., 1991) and R7 (Bettler et al., 1992; Lomeli et al., 1992) known as kainate receptors. They exhibit a low sequence homology to the first four members of the glutamate receptor family. The third class has only one member, KA-1 (Werner et al., 1991) and it has a high affinity for kainate but in contrast to the other cloned kainate receptors, domoate is much less potent at this receptor than kainate. In addition, a metabotropic receptor coupled to a G protein has been isolated and sequenced (Masu et al., 1991; Houamed et al., 1991). Very recently, Sun et al. (1992) have reported the identification of two glutamate receptor complementary deoxyribonucleic acids (DNA) of the KA subtype in human brain tissue.

Na⁺
Ca²⁺ Asp
Glu
Spermine
Zn²⁺ Gly
NMDA
(Ca²⁺)
Na⁺
PCP
Mg²⁺
Glu
Glu
AMPA
Glu
AMP/GMP
L-AP4 G PDE
cAMP/cGMP
Ca²⁺
IP₃
G PLC DAG
ACPD PIP₂
Glu
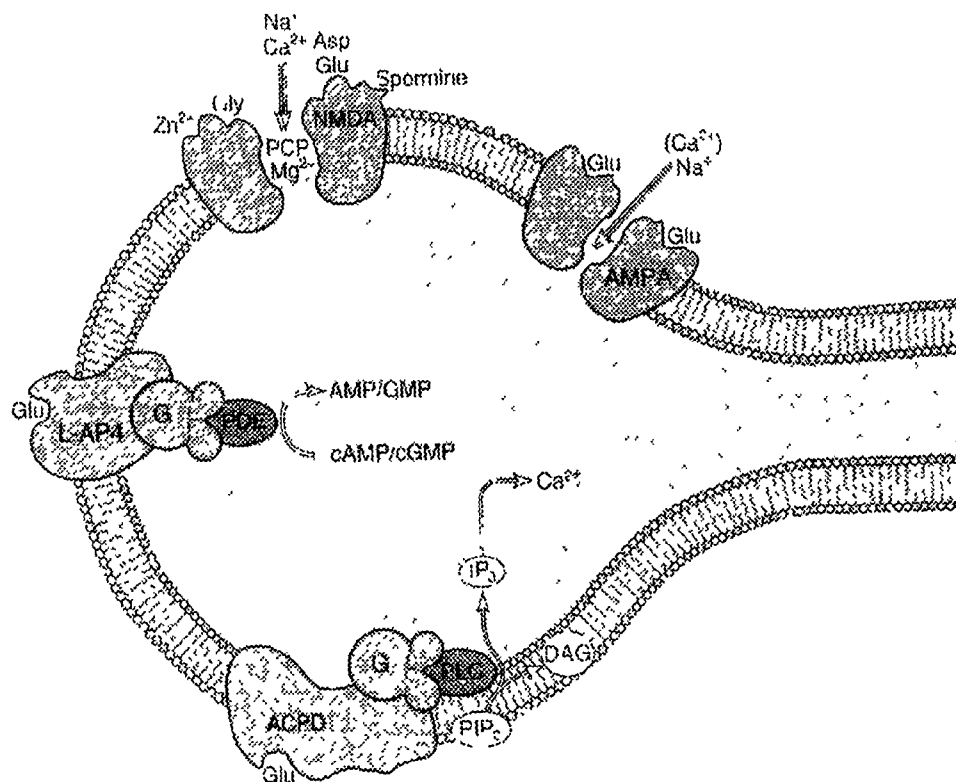
Figure 1. - Molecular views of four types of glutamate receptors.

Two heteromeric ionotropic receptors are shown, the NMDA and AMPA receptors, and two metabotropic receptors, the L-AP4 and ACPD receptors. The NMDA receptor channel is blocked by Mg2+ and phencyclidine (PCP). Zn^{2+} is a negative modulator, and polyamines such as spermine are positive modulators, of NMDA channel activation. Both L-AP4 and ACPD receptors are metabotropic in that they are coupled via G proteins (G) to intracellular enzymes, phospholipase C (PLC) for the ACPD receptor, and phosphodiesterase (PDE) for the L-AP4 receptor. (redrawn from Dingledine and McBain, 1994).

Conspicuously absent from these early reports was the cloning of the NMDA receptor. This is despite the fact that many academic and pharmaceutical laboratories and millions of dollars in research money was focused on the . cloning of the NMDA receptor. However, late in 1991, the protein sequence of a functional NMDA receptor (NR1), "the holy grail of neurotransmitter receptor molecular neurobiology" (Mayer, 1991) was at last published in Nature (Moriyoshi et al., 1991). Interestingly, in the same Nov. 7 1991 issue, another group reported the sequence of an entirely different glutamate binding subunit of an NMDA receptor (Kumar et al., 1991). However, due to various problems with the methodology used by the Michaelis group (see Barnard, 1992) and the extensive characterization of the NR1 subunit by Nakanishi's group, there is significantly more evidence to support NR1 (Moriyoshi et al., 1991) as the first cloning of an NMDA receptor subunit. The molecular weight of the NR1 subunit is 105.5 K and it contains a large N-terminal portion and 4 transmembrane domains (Moriyoshi et al., 1991). It exhibits 22-26% homology with the previously cloned AMPA/KA receptor subunits and the similarity is especially notable in the transmembrane domains (Moriyoshi et al., 1991).

NR1 was characterized by injecting Xenopus oocytes with mRNA derived from the cloned receptor (plasmid cDNA pN60). The addition of 100 μM NMDA to oocytes cultured in Mg^{2+}-free, glycine-supplemented medium caused an inward Ca^{2+} current. The electrochemical response to NMDA was significantly reduced by the removal of glycine. They also demonstrated that the NR1 subunit could be blocked by Mg^{2+} in a voltage dependent manner (i.e. Mg^{2+} blocked NMDA response at holding potentials between -80 mV and -30 mV and the block was removed at potentials more positive than -20 mV). The glutamate agonists (in order of potency): L-glutamate, NMDA, homocysteate (HCA), ibotenate and quisqualate induced a significant electrophysiological response. However,

kainate, AMPA, ACPD and GABA had no effect. Competitive antagonists of the NMDA receptor (APV, CPP and CGS 19755) reduced the electrophysiological response of transfected oocytes to NMDA by 50 to 70%. The non-competitive antagonists MK-801 and Zn^{2+} reduced the response to NMDA by 93% and 78%, respectively. Mayer (1991) states that "the homomeric NMDA receptors behave in a remarkably similar manner to NMDA receptors in intact tissue."

As predicted, the report by Nakanishi's group set the stage for the discovery of three other complementary DNA species encoding NMDA receptor subunits NMDAR2A (NR2A), NR2B and NR2C (Meguro et al., 1992; Monyer et al., 1992; Kutsuwada et al., 1992). The three new members of the NMDA receptor subfamily have a molecular mass of approximately 150 K. They are 55 to 70% homologous to each other but exhibit less than 20% sequence similarity to NR1. Nakanishi's NR1 is expressed in neuronal cells throughout the CNS, with particularly high levels in the hippocampus, cerebral cortex and cerebellum (Moriyoshi et al., 1991). Interestingly, the newly discovered subunits (NR2A, 2B and 2C) are much more restricted in their distribution and have different patterns of distribution in the brain. For instance, NR2A is present in the forebrain and cerebellum, NR2B is also found in the forebrain but NR2C expression is found exclusively in the cerebellum. Monyer et al. (1992) noted that the hypothalamus only contains the NR1 transcript and suggests there must be additional NMDA receptor subunits.

Alone or in combinatione with one another, the NR2 subunits do not form functional ion channels (Monyer et al., 1992). Forrest et al. (1994) generated mice which carried a disrupted NR1 allele and showed that in embryonic cell cultures, NMDA does not alter the calcium current in the mutant mice, illustrating that the function of NR1 cannot be replaced by other subunits of the NMDA receptor. The electrophysiological response to NMDA was also examined in oocytes

transfected with NR1 and any one of the NR2 subunits. The heteromeric channels generated currents which were 100 times larger than those observed by Moriyoshi et al. (1991) with homomeric NR1 channels. In addition, the various combinations of NR1 and the NR2 subunits in the oocyte expression system had different functional characteristics. For example, they differed in the degree to which glycine co-agonist is required, the sensitivity to Mg^{2+} blockade and the extent to which binding and voltage are required for operation.

Monyer et al. (1992) believe that they have provided appropriate molecular and functional evidence for different NMDA receptor subtypes being generated by heteromeric channel assembly of the NR1 subunit with members of the NR2 subunit family. Indeed the existence of different subtypes of NMDA receptor has already been reported *in vivo* (Cotman et al., 1988; Perkins and Stone, 1983; Williams et al., 1993). The diverse heteromeric NMDA receptor model proposed by Monyer et al. raises the possibility that NMDA receptors with different physiological properties may be differentially localized in the CNS with cell-specific functions. Stevens (1992) states that "with the identification of these additional NMDA receptor subunits it now becomes possible to understand the molecular mechanisms responsible for the NMDA receptor's rich repertoire of characteristics".

This field continues to develop quickly. A recent review article has identified 54 glutamate receptor genes cloned since 1989 (Hollmann and Heinemann, 1994).

Developmental Expression of EAA Receptors

Several recent studies have examined the ontogenesis of excitatory amino acid (EAA) receptors in various areas of the brain (McDonald et al., 1990b; Pellegrini-Giampietro et al., 1991; Tremblay et al., 1988; LoTurco et al., 1991; Represa et al., 1986; Erdo and Wolff, 1989). The differential expression of EAA receptors paralleling periods of active synaptogenesis and stabilization of synaptic connections implicates EAA receptors in the control of brain development.

(a) NMDA Receptor:

McDonald et al. (1990b) studied the developmental pattern of expression of three components of the NMDA receptor complex (NMDA, glycine and phencyclidine (PCP) recognition sites). Peak densities of the NMDA recognition site (50 to 120% above adult levels) occurred between postnatal day (P) 10 and P28. In contrast, the developmental increases in the PCP and glycine sites were delayed relative to expression of the NMDA recognition site as maximal binding densities were not reached until P28 in these sites. The authors note that the developmental expression of the NMDA receptor complex in the hippocampus is in accordance with synaptogenesis, afferent lamination and the functional development of the hippocampus. Tremblay et al. (1988) also examined the density of binding sites in rat hippocampus using autoradiography. This group obtained similar results to McDonald et al. (1990). There was a change in the density of NMDA binding sites during development but they showed that the peak binding density occurred much earlier (P8) than that observed by McDonald's group.

Using patch-clamp recording LoTurco et al. (1991) have studied the initial expression of NMDA receptor associated channels in the neocortex of rats. They showed that migrating neocortical neurons do not express NMDA channels.

However, at the end of the migration and the beginning of neurite outgrowth, NMDA channels become functional. Cells in the cortical plate of embryonic P9 - 12 rats responded to NMDA with an inward current. The percentage of patches which showed NMDA channel activity gradually increased from 25% in embryonic cells to 100% in P9-12 cells (LoTurco et al., 1991). The timed appearance of NMDA channels and their increasing density in the cortex suggests a role for NMDA receptors in the development of dendrite elaboration and the establishment of synaptic contacts. In contrast, NMDA receptors in the developing dentate gyrus are reported to attenuate differentiation by decreasing cell death and birth (Gould et al., 1994).

Granule cells in the cerebellum are glutamatergic and possess NMDA receptors (Garthwaite et al., 1986). Pearce et al. (1987) reported that neurite outgrowth in cultured cerebellar granule cells is regulated by endogenous glutamate acting through NMDA receptors. Cambray-Deakin et al. (1990) have also shown that there is a developmental increase in the density of NMDA binding sites in the cerebellum followed closely by rising, although lower, levels of glycine binding sites. They noted that L-[^{3}H]-glutamate binding was much greater in the granule layer than in the external germinal layer or the molecular layer of the rat cerebellum and suggest that NMDA receptors may mediate neurite outgrowth in the granule cell layer *in vivo* as it does in culture (Cambray-Deakin et al., 1990).

In the wake of the NMDA.R cloning, (Moriyoshi et al., 1991) several groups have examined the developmental expression of the gene coding for this receptor. In whole brain preparations, mRNA of the NR1 subunit of the NMDA.R is low in neonatal rats and rises progressively from P14 to P21 (Riva et al., 1994). More specifically, NR1 mRNA has been reported to peak in expression in cerebellum, hippocampus and midbrain/diencephalon on P60, P10 and P10,

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respectively (Pujic et al., 1993). Despite the use of the same cDNA probe, Franklin et al. (1993) reported that NMDA.R1 mRNA levels in frontal cortex and hippocampus increased 3-fold between P3 and P15 and doubled between P15 and P67. However, in agreement with Pujic et al. (1993), this group also reported steady state levels in the cerebellum between P3 and P15 and doubling of NMDA.R1 mRNA content between P15 and P67. Even greater complexity/diversity is introduced with a report of developmental differences in the expression of different splice variants of the NMDA.R1 gene (Laurie and Seeburg, 1994; Della Vedova et al., 1994).

The expression of the NR2 subunits (A,B, C and D) of the NMDA receptor also appears to be developmentally regulated (Monyer et al., 1994). Although NR2C mRNA is not evident in the adult hippocampus using *in situ* hybridization, there is a period between P7 and P14 where specific areas of the hippocampus express significant amounts of NR2C mRNA suggesting a potential role for NR2C in neuronal development (Pollard et al., 1993). Although the NR2A and NR2B subunits are expressed equivalently and at high levels in the adult hippocampus, the expression of the NR2B subunit appears to be dominant in the neonatal (up to P8) animals (Riva et al., 1994). The developmental regulation of the various NMDA receptor subunits may explain functional heterogeneity in NMDA receptors in juvenile rats. Williams et al. (1993) using binding assays with rat brain membranes have reported a high affinity for the NMDA antagonist, ifenprodil, in neonatal rat brains which is significantly reduced (100-fold lower affinity) compared to membrane preparations from adults. Interestingly, the change in affinity for ifenprodil could be explained by the combination of NR1 with different NR2 subunits. For instance, in *Xenopus* oocytes injected with NMDA receptor subunit mRNA, the

response of NR1 and NR1/NR2B, but not NR1/NR2A receptors, were inhibited by application of ifenprodil (Williams et al., 1993).

(b) *Non-NMDA Receptors:*

There is also evidence for a developmental increase in the density of non-NMDA glutamate receptors in various regions of the brain. Kainate and AMPA (quisqualate) receptors show an increased density of binding sites in the developing rat cortex (Erdo and Wolff, 1989). Using whole tissue homogenates to study ligand binding of [^{3}H]-kainate and [^{3}H]-AMPA, Erdo and Wolff (1989) characterized the density of binding sites and the binding affinities for these two ligands in the cortex of rats aged 2 to 360 days. There appears to be high levels of AMPA/kainate binding on P2, reaching a maximum at 6 days and progressively dropping to adult levels by three to four weeks. There is also a difference in binding affinity for AMPA in juvenile (P6; K_d=148 ± 14 nM) compared with adult brains (P180; K_d=67 ± 9 nM). The ontogeny of kainate and AMPA binding in the cortex does not seem to correspond with the period of increased excitatory input suggesting a trophic rather than a tropic action of glutamate in the cortex at this age.

Binding sites for kainate and quisqualate increase developmentally in both the granule layer and the molecular layer of the cerebellum (Cambray-Deakin et al., 1990). As well, Represa et al. (1986) showed using autoradiography that there is a transient appearance of high density kainate binding in the human fetus (25 week gestation and newborns). The developmental expression of three genes encoding non-NMDA glutamate receptors (GluR1, R2, R3) has been studied using *in situ* hybridization in the striatum, cortex, hippocampus and cerebellum of rats (Pellegrini-Giampietro, 1991). They reported that there was both a spatial and temporal variation in the expression of all three glutamate receptors between P2 and P80. The developmental regulation of glutamate gene expression in rat

brain suggests that non-NMDA glutamate receptors may have a role in developmental plasticity.

The cloning of the glutamate receptors has significantly added to the literature on the developmental profile of non-NMDA receptors as well as NMDA receptors. Generally, the kainate receptor subunit genes (GluR5, 6, 7, KA-1 and KA-2) appear to peak in expression in the late embryonic or neonatal period of the developing rat brain (Bahn et al. 1994). Condorelli et al. (1992) reported constant high levels of metabotropic glutamate receptor (mGluR) mRNA in the hypothalamus and olfactory bulb from P1 to adult rats. In contrast, mGluR1 mRNA and protein expression in the hypothalamus has been quantified by van den Pol et al. (1994) and shown to increase from E18, peak between P10 and P20 and diminish to adult levels. Interestingly, the latter developmental trend parallels the study by Sortino et al. (1991) and Condorelli et al. (1992) showing the formation of high levels of second messenger (inositol phosphate) upon stimulation of the mGluR with ACPD in the first 8 to 10 days of life and declining progressively to negligible levels in the adult.

The differential ontogenic expression and electrochemical properties of the various components of the NMDA and non-NMDA receptors suggests that the glutamate receptors are an integral part of the mechanism regulating neuronal development in many areas of the brain including the hypothalamus.

Glutamate and Neurotoxicity

In addition to its widespread physiological effects at a variety of receptor types, glutamate is capable of inducing neuronal injury or death through a process of excessive cellular stimulation ('excitotoxicity'). Analogues such as NMDA, KA and domoic acid are even more potent than glutamate itself. A full description of this large field is beyond the scope of this thesis but has been

adequately reviewed elsewhere (Seress, 1982; Olney and Price, 1983). Glutamate is also thought to be responsible for neuronal injury in certain neurological diseases (Lipton and Rosenberg, 1994).

Much work has been done in the use of EAA as tools to probe the organization and function of the neuroendocrine system (for reviews see Olney and Price, 1978; 1983). Glutamate neurotoxicity results from sustained activation of NMDA-gated ion channels resulting in a prolonged influx of Ca^{2+} ions causing neuronal degeneration and cell death (Choi, 1987; Garthwaite and Garthwaite, 1989). Brain regions known collectively as circumventricular organs (CVOs; see Fig. 2) are particularly susceptible to damage from monosodium glutamate (MSG; Olney, 1969). Neurons in the ARCN/median eminence (ME) area are vulnerable but damage induced by EAAs has also been reported in subfornical organ (SFO) and area postrema (AP) (Olney and Price, 1978). Glial cells and axonal processes in the ARCN appear to be spared from the neurotoxic effects of MSG (Lemkey-Johnston and Reynolds, 1974; Simson et al., 1977). More recently, Meister et al. (1989) have provided evidence for MSG-induced changes in a variety of neurochemically-identified cell populations in rat ARCN/ME.

MSG treatment of neonatal rats produces varying results on the onset of puberty. Dawson et al. (1989) and Bakke et al. (1978) observed no changes in the mean day of VO which contrasts with the delay in puberty reported by Rodriguez-Sierra et al. (1980). In our laboratory, MacDonald and Wilkinson (1990) reported MSG-induced acceleration of puberty. Interestingly, the advancement of puberty was inversely related to the dose of neonatal MSG administered (MacDonald and Wilkinson, 1990) which may explain the discrepancy between published studies.

Neonatal stimulation of hypothalamic glutamate receptors with high doses of MSG (eg. 4 mg/g; P2, P4, P6, P8) results in severe endocrinopathies in

the adult (Bakke et al., 1978) most likely caused by destruction of specific neurons in the ARCN of treated rats (Meister et al., 1989). Endocrinologic abnormalities have been reported in the hypothalamic-pituitary-gonadal (H-P-G) axis (Bakke et al., 1978; Nemeroff et al., 1978b, 1981; Greeley et al., 1980; Dyer et al., 1981), H-P-adrenal axis (Olney and Price, 1978; Dolnikoff et al., 1988), and H-P-thyroid axis (Nemeroff et al., 1978a; Greeley et al., 1980). The role of glutamate excitotoxicity in developmental neuropathology has been recently reviewed by Olney (1993).
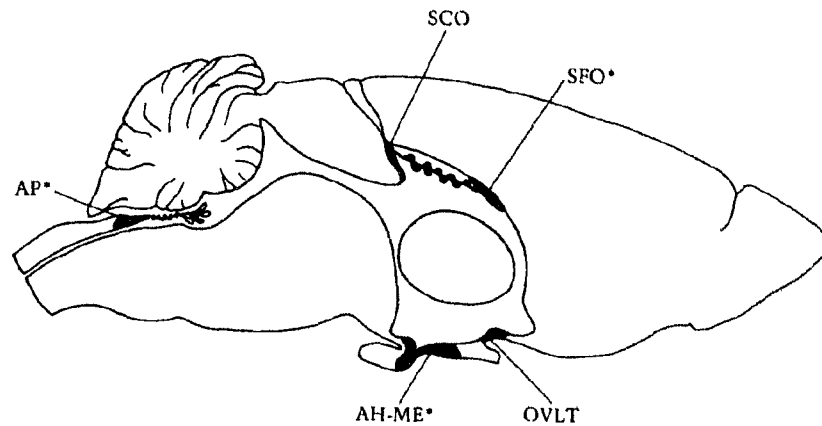
Figure 2. Diagram of midsaggital section of rat brain showing the location of the circumventricular organs.

Abbreviations: AP, area postrema; SCO, subcommissural organ; SFO, subfornical organ; OVLT, organum vasculosum of the lamina terminalis; AH-ME, arcuate-median eminence region of the hypothalamus. *Asterisks indicate regions most vulnerable to damage by systemically administered excitotoxins, such as kainic acid (redrawn from Olney and Price, 1978).

Glutamate and Synaptogenesis

Although glutamate is neurotoxic when administered in high doses (Seress, 1982; Meister et al., 1989), low doses of glutamate can promote cell survival and modulate the structure of developing neurons (Aruffo et al., 1987). MSG (5 mM) applied to whole-brain dissociated cultures of 15 day-old rat fetuses increases neuronal size, volume, the number of synaptic vesicles, the number of mitochondria, development of rough endoplasmic reticulum and the number of synapses. This mode of MSG treatment seems to have accelerated neuronal differentiation (Aruffo et al., 1987).

The effect of EAA on cell differentiation and neurite outgrowth has also been examined in cultures of cerebellar granule cells. These neurons release glutamate and possess NMDA receptors neurite outgrowth in these cells can be blocked by enzymatic removal of endogenous glutamate, and by pharmacological blockade of glutamate receptors by kynurenic acid and 2-amino-5-phosphopentanoic acid (APV; Pearce et al., 1987). NMDA has trophic effects on them (Balazs et al., 1988). Moran and Patel (1989) showed that glutaminase (an enzyme in the pathway which synthesizes glutamate) activity was elevated in a time and dose-dependent manner after NMDA treatment of cultured cerebellar granule cells. The increase in glutaminase activity was blocked by APV, MK-801 and Mg^{2+}. These studies demonstrate that EAA can induce morphological and biochemical differentiation of cerebellar neurons.

There is some evidence that glutamate deleteriously affects cell survival of cultured hippocampal neurons (Mattson and Kater, 1989). It seems to be the result of using high doses of MSG (1 mM), since lower doses of glutamate can enhance outgrowth of processes from hippocampal neurons (Mattson et al., 1988). Also, in cultured dentate granule cells, NMDA promotes dendritic branching of neurons. Branching is reduced by application of MK-801 but this

antagonist promotes an increase in the mean length of each branch (Brewer and Cotman, 1989). In these experiments EAAs seem to contribute to neuronal morphogenesis by increasing dendritic branching.

Cornell-Bell et al. (1990) demonstrated that glutamate induces an increase in the number of filopodia on the surface of cultured hippocampal astrocytes. Glutamate, kainate, and quisqualate but not NMDA increased the mean number of filopodia on the surface of astrocytes compared with controls. Therefore, only non-NMDA receptors seem to be involved in filopodia formation. Neuronal filopodia are very important in formation of adhesive connections and may even develop into mature axons capable of forming synapses (Tsui et al., 1985).

Additional evidence implicating NMDA in neuronal plasticity is provided by Brooks et al. (1991). NMDA treatment increased synapse formation in the occipital cortex of postnatal rats. Electron microscopy (EM) studies showed that an i.p. injection of NMDA increased the length of synaptic contacts in juvenile and pubertal male rats. In young male rats (P8) NMDA administration increased synaptic density. Once again, these data stress the importance of the NMDA receptor in synaptogenesis and its overall contribution to neuronal plasticity.

The Effects of Glutamate on Cell Growth and Survival

Although excess activation of glutamate receptors causes neuronal death (Choi, 1987; Foster et al., 1988; Mattson et al., 1988) this depends on the concentration of glutamate or its agonists as there is a continuum of responses ranging from promotion of cell survival at low doses to cell death at high doses. Balazs et al. (1988, 1990) demonstrated that NMDA and KA promote the survival of cultured cerebellar granule cells. NMDA seemed to be maximally effective at a high dose of 100 mM whereas KA induced a biphasic response, promoting survival at low doses (15-50 mM) and compromising survival at high doses (500

mM). The same group demonstrated that KA-enhanced survival of granule cells is potentiated by the antagonists APV and MK-801. The authors hypothesized that KA has a dual role in the cell acting both as a trophic and a toxic agent. The trophic effects of KA seem to be mediated by the KA receptor and the toxic effects via a NMDA-like glutamate receptor (Balazs et al., 1990).

NMDA receptors in spinal cord dorsal-root ganglion cultures also regulate cell survival (Brenneman et al., 1990). Interestingly, blockade of NMDA receptors by AP-5 had a similar biphasic response to glutamate. Low concentrations of AP-5 (0.1 mM) increased cell survival whereas high concentrations (100 mM) caused cell death. In contrast to the survival-promoting effects of NMDA on granule and spinal cord neurons, neocortical explants seem extremely sensitive to NMDA (Ruijter and Baker, 1990). An optimal concentration of 10 mM of NMDA enhanced cell survival whereas doses of 20 mM or above were neurotoxic.

NMDA receptors may rescue neurons from death following head injury. CNS injuries sustained in motor vehicle accidents, falls and assaults are the result of rapid acceleration or deceleration of the brain. Lucas and Wolf (1991) have developed a model of rapid acceleration injury (RAI) where multiple impacts are delivered tangentially to the growth plane of cultured spinal cord neurons *in vitro* (Lucas and Wolf, 1991) leading to neuronal damage and death. Ketamine, a non-competitive NMDA-antagonist, prevents cell death and morphological changes such as somal swelling and nuclear prominence in cultures of "injured" spinal cord neurons (Lucas and Wolf, 1991). The author's demonstrated that the sensitivity of the cultured neurons to RAI is proportional to the density of NMDA receptors and proposed that cell death may be initiated by Ca^{2+} leakage through the NMDA receptor-associated ion channels.

It is clear that the effects of EAA (NMDA, KA) on cell survival are largely dependent on the dose, the locus in the brain, and the age of the experimental animals or neuronal cultures. The differential properties of glutamate are exerted via its three receptor subtypes enabling EAA receptors to promote neuronal plasticity, cell survival or death.

The Effects of EAA on the Cytoskeleton

Neuronal growth and differentiation can be broken down into periods of neurite outgrowth, establishment of neuronal direction, synaptogenesis and eventually stabilization of these processes in a mature neuronal format. Glutamate may influence neurite outgrowth and stability by directly acting on microtubule associated proteins (MAPs). MAPs are integral components of the cytoskeleton that have been implicated in neurite outgrowth, plasticity and maintenance of neuronal morphology (Matus, 1988; Tucker, 1990). MAPs crosslink microbutules to one another and to other cytoskeletal components (Matus, 1988) and they display different spatial distributions in the brain, i.e. MAP2 is found in dendrites and cell bodies (Bernhardt and Matus, 1984) whereas tau proteins are found exclusively in axons (Binder et al., 1985).

Bigot and Hunt (1990) examined the effects of EAA on the localized expression of tau and MAP2 in cultured cortical and spinal neurons. In control conditions, tau-immunoreactivity (-ir) was restricted to axons. However, stimulation with NMDA, glutamate, kainate or quisqualate resulted in enhanced tau-ir in axons and the *de novo* appearance of tau-ir in dendrites and cell bodies. This effect was blocked by appropriate antagonists. In addition, fifteen minutes after stimulation with NMDA, kainate, or quisqualate, MAP2 staining was strikingly arranged in a network of filamentous bundles (Bigot et al., 1991) in cultures of cortical neurons. It is possible that glutamate agonists may influence

synaptogenesis by directly altering the expression and distribution of MAPs in neurons.

MAP2 has over 20 phosphorylation sites and its function is highly correlated with the degree of phosphorylation (Theurkauf and Vallee, 1983). *In vitro* studies indicate that MAP2 phosphorylation decreases its affinity for tubulin, resulting in decreased microtubule formation (Yamamoto et al., 1983). Halpain and Greengard (1990) have demonstrated that glutamate acting preferentially through NMDA receptors stimulate the dephosphorylation of MAP2. NMDA may play a significant role in determining dendritic morphology by altering distribution of MAP2 in the cell and by altering its ability to promote microtubule assembly.

Glutamate and Plasticity in Developing Systems

Glutamate and its receptors play important roles in neuronal survival, cellular differentiation, synaptogenesis and neurite stability. The role of EAAs as trophic factors has been well documented in many isolated sites in the brain. EAA receptors also appear to play a critical role in the organization of developing systems such as the kitten visual cortex and the optic tectum of the frog.

The development of the kitten visual system is readily modified by visual experience during the second and third month after birth (Kleinschmidt et al. 1987). Monocular deprivation of kittens leads to a rapid disconnection of excitatory input to the deprived eye. Development seems to depend on afferent fibers being guided to their proper targets in the visual cortex and the establishment of stable synaptic connections. Experiments with the glutamate agonist, NMDA, suggest that glutamate is involved in the experience-dependent

modifications in the kitten visual cortex (Gu et al., 1989; Kleinschmidt et al., 1987; Rauschecker and Hahn, 1987).

Kleinschmidt et al. (1987) used osmotic minipumps to administer the NMDA antagonist, APV, to the striate cortex of kittens during monocular deprivation. Animals treated with APV did not display the ocular dominance of saline-treated monocular deprived kittens, suggesting that an NMDA receptor dependent process may be responsible for the trimming of synaptic connections during the critical period. Conversely, Gu et al. (1989) reversed monocular deprivation and demonstrated that chronic blockade of NMDA receptors significantly reduced the recovery of neuronal responses to the newly opened eye. Therefore, NMDA receptors may be required for strengthening of synaptic connections during the neuronal response to visual experience.

The NMDA receptor also seems to be involved in regulating plasticity in the optic tectum of the developing three-eyed frog (*Rana pipiens*). The retinal ganglion cells (RGC) from the three eyes project to the same optic tectum where the terminals segregate into stereospecific ocular dominance stripes. RGC activity is required for the establishment and maintenance of a topographic projection within the developing or regenerating retinotectal system (Reh and Constantine-Paton, 1985; Stryker and Harris, 1986; Dubin et al., 1986; Cline and Constantine-Paton, 1990). Sustained application of the NMDA antagonists, APV or MK-801, resulted in the desegregation of the RGC terminals in the optic tectum (Dubin et al., 1986; Cline and Constantine-Paton, 1990). APV-treated tadpoles also showed a 35% reduction in RGC branch density (Cline and Constantine-Paton, 1990). Conversely, NMDA treatment produces ocular dominance stripes with sharp, defined borders and increases arbor density by approximately 50% (Reh and Constantine-Paton, 1985; Cline and Constantine-

Paton, 1990). The data demonstrate that the NMDA receptor is involved in the activity-dependent structural refinement of optic tectum.

V. NMDA and Pituitary Hormone Secretion

Luteinizing Hormone and Follicle Stimulating Hormone

The large body of evidence implicating the excitatory neurotransmitter glutamate and its NMDA receptor in the regulation of LH and FSH secretion has recently been reviewed by Brann and Mahesh (1994). *In vivo* treatment with NMDA increases serum LH levels in the rat (Price et al., 1978a,b; Olney and Price, 1980; Schainker and Cicero, 1980; Johnson et al., 1985; Urbanski and Ojeda, 1987; Arslan et al., 1988; MacDonald and Wilkinson, 1990), hamster (Urbanski, 1990; Meredith et al., 1991; Hui et al., 1992; Urbanski, 1992), sheep (Ebling et al., 1990; Estienne et al., 1990; Jansen et al., 1991; l'Anson et al., 1993), and monkey (Wilson and Knobil, 1982; Gay and Plant, 1987, 1988; Claypool and Terasawa, 1989; Medhamurthy et al., 1992; Gay et al., 1993). In the juvenile rat, a subcutaneous injection of NMDA causes a rapid (maximal 7.5 min. post-injection) and transient (return to basal levels 30 min. post-injection) increase in serum LH levels (male: Olney et al., 1976; Olney and Price, 1980; female: MacDonald and Wilkinson, 1992). In contrast, the non-competitive NMDA receptor antagonist, MK-801, reduces pituitary LH secretion (Urbanski and Ojeda, 1990; Veneroni et al., 1990; MacDonald and Wilkinson, 1992). Cocilovo et al. (1992) reported a significant decrease in LHRH-like immunoreactivity (-ir) and LHRH mRNA in the hypothalamus of MK-801-treated peripubertal rats. Phencyclidine (PCP) antagonizes the action of EAA at the NMDA receptor and cerebroventricular injection of PCP reduces the ability of glutamate, homocysteic acid and castration to elevate serum LH levels (Boggan and Ondo, 1989). Recently, Petersen et al. (1992) have used *in situ* hybridization to detect an

increase in LHRH mRNA in cells at the level of the organum vasculosum of the lamina terminalis (OVLT) and the rostral POA in the adult male rat 15 min. after an injection (i.v.) of NMDA. Due to the temporal correlation between NMDA-evoked LH release and the NMDA-induced increase in LHRH mRNA, Petersen et al. (1992) suggested that NMDA may simultaneously activate parallel neuronal pathways. Gore and Roberts (1994) have replicated these results using an RNase protection assay and have shown that NMDA appears to increase LHRH mRNA post-transcriptionally, most likely through an increase in mRNA stability. Liaw and Barraclough (1993) have reported that, in contrast to estrogen-treated ovariectomized control rats, NMDA stimulation reduces LHRH mRNA in androgen-sterilized rats.

In addition to its involvement in the tonic release of LH, the NMDA receptor has a physiological role in the production of LH pulses (Arslan et al., 1988; Bourguignon et al., 1989b) and the generation of the first proestrous surge of LH in the rat (Meijs-Roelofs et al., 1991). Endogenous pulses of LHRH released from hypothalamic explants *in vitro* are completely abolished by MK-801 (Bourguignon et al., 1989b). In castrated male rats, treatment with the competitive NMDA antagonist AP-5 suppresses pulsatile LH release as well as the baseline level of LH secretion (Arslan et al., 1988). Meijs-Roelofs et al. (1991) reported that chronic blockade of the NMDA receptor with MK-801 delays but does not prevent first ovulation, whereas acute treatment blocks the proestrous LH peak. Lopez et al. (1990) found that intraventricular administration of the NMDA antagonist, AP-7, completely blocked the estradiol-induced LH surge in ovariectomized rats. Brann and Mahesh (1991a,b) also found that pretreatment with the noncompetitive NMDA antagonist, MK-801, blocked the progesterone-induced LH and FSH surge in estrogen-primed ovariectomized rats.

The site of action of NMDA is believed to be suprapituitary due to the fact that *in vitro* exposure of rat and primate pituitaries to NMA does not stimulate LH release (Tal et al., 1983), and NMDA-induced LH release is completely blocked by pretreatment with an LHRH antagonist (rat: Cicero et al., 1988; hamster: Urbanski, 1990). The traditional belief is that NMDA enhances LH secretion by acting on hypothalamic glutamate receptors to induce the release of LHRH (Ondo et al., 1988; Cicero et al., 1988; Claypool and Terasawa, 1989; Plant et al., 1989). However, perifused rat pituitary cells *in vitro* release anterior pituitary hormones, including LH, in response to EAA stimulation (Zanisi et al., 1991, 1994; Lindstrom and Ohlsson, 1992; Login, 1990). In addition, NMDA receptor mRNA has recently been localized to the anterior pituitary (Brann et al., 1993a). The site of action of NMDA on LH release has recently been questionned.

Despite this new evidence, the hypothalamus is commonly accepted as the major site of EAA action but the precise neuronal locus of this effect is controversial. Several researchers have attempted to establish the site of EAA action by measuring LH release after infusion into specific nuclei in the brain and by examining the ability of glutamate and its specific agonists to stimulate the release of LHRH from fragments of the hypothalamus *in vitro*. Ondo et al. (1988) found that infusion of NMDA into the medial POA increased pituitary LH secretion 2-7 fold over controls but infusion into the anterior hypothalamic nucleus, ventromedial hypothalamic nucleus or the arcuate nucleus had no effect. These results are supported by the findings of Donoso et al. (1990) and Lopez et al. (1992) showing that non-NMDA receptor agonists were the most potent stimulators of LHRH release from nerve terminals in ARCN-ME fragments *in vitro*. The stimulatory action of glutamate on LHRH release was not blocked by the NMDA receptor antagonist, AP-7 suggesting that "non-NMDA

receptors are primarily involved in EAA-induced LHRH release at the level of ARCN-ME". (Lopez et al., 1992). Contrary to these findings, Bourguignon et al (1989a) treated mediobasal hypothalamus (MBH) fragments from immature male rats *in vitro* with NMDA and triggered the rapid release of LHRH. However, the dose used by Bourguignon et al. was very large (50 mM) and the results may not be relevant to a physiological mechanism in the hypothalamus. Clearly this question of the site of action of NMDA needs to be investigated further.

The ability of NMDA to stimulate LH and LHRH release may be age-dependent. In male rats, Cicero et al. (1988) demonstrated that NMDA-stimulated LH release is maximal between P20 and P35 with minimal LH response in the neonate (P10, P15) or young adult (P40, P60). This "bell-shaped" pattern has also been reported in female rats (MacDonald and Wilkinson, 1990). Maximal LH secretion was observed between P15 and P28 with a dramatic drop in LH responsiveness by P30 indicating that NMDA-induced LH secretion declines immediately prior to the onset of puberty.

Bourguignon's group has examined the ontogeny of pulsatile LHRH secretion in hypothalamic explants. It is well known that the nocturnal pattern of pulsatile LH secretion observed in the peripubertal rat is a gonadal independent event. Orchidectomy does not prevent the age-related changes in the sensitivity of the NMDA receptors involved in LHRH secretion (Bourguignon et al., 1992a).

Consistent with what has been observed *in vivo* (Cicero et al., 1988; MacDonald and Wilkinson, 1990), the dose of MK-801 required to suppress pulsatile LHRH secretion is lower in the male rat at 25 days than at 50 days of age, indicating that there is a prepubertal activation of NMDA receptors (Bourguignon et al., 1990a). Bourguignon et al. (1990b) have proposed that the peripubertal increase in the frequency of pulsatile LHRH secretion may be due to a decrease in NMDA receptor sensitivity in the hypothalamic pulse generator.

This postulate may explain the observed decrease in NMDA-induced LH secretion prior to the onset of puberty (MacDonald and Wilkinson, 1990). The lack of activation of pulsatile LHRH secretion in hypothalamic explants from young male rat brains (P15) may indicate a strong NMDA receptor-mediated inhibition (Bourguignon et al., 1992b). For instance, administration of a low-dose of MK-801 to hypothalamic explants of P15 male rats increases the frequency of pulsatile LHRH secretion and *in vivo* treatment of immature male rats with MK-.801 leads to precocious sexual maturity.

Estradiol and NMDA-Induced LH Secretion

It is clear that in the majority of instances, NMDA has a stimulatory effect on gonadotropin release in normal animals. However, variations in serum steroid levels have been shown to modulate its effects. For instance, Pohl et al. (1989) demonstrated that the LH response to NMDA is not evident in lactating rats. In contrast, Carbone et al. (1992) illustrated that NMDA-evoked LH and FSH release is tremendously potentiated in prepubertal rats pre-treated with estradiol benzoate and progesterone. Luderer et al. (1993) compared the LH response to NMA in cycling rats during metestrous (low estrogen) and proestrous (high estrogen) but failed to detect a difference in the degree of NMA-stimulated LH release. However, Reyes et al. (1991) noted that in normal adult rhesus monkeys, the effects of NMA on LH release were greatest during the luteal phase of the cycle and were absent in the early follicular phase of the cycle when steroid levels are low (Reyes et al., 1991).

The presence of circulating estradiol also appears to have differential effects on the role of the NMDA receptor in gonadotropin release. For example, Brann and Mahesh (1992) and Luderer et al. (1993) reported that NMDA suppresses serum LH levels in ovariectomized rats, whereas, it simulates LH

release in estrogen-primed ovariectomized rats. Similarly, NMA treatment significantly reduces LH secretion in long-term ovariectomized rhesus monkeys and steroid replacement prevents the NMA-induced decrease in LH (Reyes et al., 1990, 1991). The observation that the neuronal response to EAA is enhanced by estradiol (Smith, 1989) may provide a possible explanation for the action of estradiol on NMDA regulated LH release. NMA has no effect on LH release in ovariectomized ewes but does stimulate LH release after estrogen replacement therapy (Estienne et al., 1990). In contrast, MacDonald and Wilkinson (1992) have reported that NMDA treatment was still able to stimulate LH release in ovariectomized prepubertal rats. The discrepancy in these findings may be due to experimental differences in the length of time between ovariectomy and NMDA treatment. In addition, the neural mechanism controlling the steroid-dependent biphasic role of NMDA in the adult may not be present in prepubertal rats.

Other Neurotransmitters and NMDA-Induced LH Release

Given the widespread distribution of glutamate in the hypothalamus (Van den Pol and Trombley, 1993), it is not surprising that several researchers have studied the possible interactions between NMDA-sensitive neuronal pathways and other neurotransmitters in the control of gonadotrophin release. GABA is the dominant inhibitory neurotransmitter in the hypothalamus (Decavel and van den Pol, 1990) and it also appears to play a role in LH release (Masotto et al., 1989; Hartman et al., 1990; Akema and Kimura, 1991; Jarry et al., 1991; Moguilevsky et al., 1991; Seltzer and Donoso, 1992). Price et al. (1978b) were the first to demonstrate that GABA can block the stimulatory effects of NMA on LH release. However, as illustrated by Donoso et al. (1992) and Akema and Kimura (1993), the interaction of the GABA-ergic system with the glutamatergic system

involved in LH release is quite complex. The $GABA_A$ receptor antagonist, bicuculline prevented the glutamate-induced release of LHRH from ARCN-ME fragments *in vitro* (Donoso et al., 1992). Interestingly, the combination of a $GABA_A$ agonist, muscimol, and glutamate had an additive stimulatory effect on LHRH secretion. However, stimulation of the $GABA_B$ receptors opposedNMDA-induced LHRH release *in vitro*. The results of GABA and glutamate interaction experiments in LH release obtained *in vitro* are different to those observed *in vivo*. Akema and Kimura (1993) found that activation of either $GABA_A$ or $GABA_B$ receptors antagonized the effects of NMDA on LH release.

There is also interaction of the NMDA-sensitive neuronal pathway controlling LH secretion with the opioid and norepinephrine (NE) systems. Cicero et al. (1988) demonstrated that both NMDA and the opioid antagonist, naloxone, release LH in peripubertal male rats. The stimulatory effects of NMDA were abolished by pretreatment with morphine (Cicero et al., 1988). MacDonald and Wilkinson (1990) have also reported that blockade of NE synthesis with the drug, U-14,624, decreased basal LH secretion and NMDA-induced LH secretion.

Prolactin

In addition to stimulating LH and FSH release, the NMDA receptor also appears to stimulate prolactin secretion. Wilson and Knobil (1982) and Gay and Plant (1987) demonstrated that prolactin levels in male and female rhesus monkeys rise rapidly after NMA treatment. This rise in prolactin was not blocked by a LHRH antagonist, indicating that the action of NMDA on prolactin and LH release is mediated by different neuronal pathways. Similarly, Lincoln and Wu (1991) have observed a dose dependent increase in prolactin secretion in rams after NMDA treatment during the long but not the short days of the

photoperiodic cycle. The stimulatory effects of pulses of NMDA (i.v.) on prolactin release have also been reported by Pohl et al. (1989) in adult male and female rats. This group later showed that in the adult cycling rat, LH but not prolactin secretion was stimulated by repetitive treatment with a nonconvulsive dose of KA (Abbud and Smith, 1991). In contrast to these reports, Jezova et al. (1991) have reported that intraperitoneal (i.p.) administration of several doses of NMDA or NMA to adult male rats did not trigger prolactin secretion. The discrepancy in these findings may be attributed to differences in the manner in which NMDA was administered (i.v. vs. i.p.) or to the dose of NMDA used in each experiment (40 mg/kg vs. 2.5 to 10 mg/kg).

The EAA antagonists, AP-7 and DNQX, both block the estradiol-induced LH surge in ovariectomized rats but neither blocks the estradiol-induced prolactin surge (Lopez et al., 1990). Pohl et al. (1989) have found that the prolactin response to NMA is dramatically altered during lactation in the rat. For instance, NMA suppressed prolactin when circulating prolactin levels were elevated (following suckling) and had no effect on prolactin when the circulating levels were low. Pohl et al. (1989) suggest that the alteration of the prolactin response to NMDA from stimulatory in normal adult rats to inhibitory in lactating rats may be explained by the dual stimulation of both prolactin-inhibiting (dopamine) and prolactin-stimulating systems by NMDA. Therefore, in normal adult rats, NMDA would primarily influence the neuronal system controlling the prolactin-releasing factor and in the pregnant rat, NMDA would primarily influence the dopaminergic system. A similar hypothesis was proposed by Arslan et al. (1991) who found that the NMA stimulated secretion of prolactin, was partially blocked by the competitive antagonist AP-5 while AP-5 administered alone evoked a significant release of prolactin.

Adrenocorticotropin Hormone

The NMDA receptor also stimulates the secretion of adrenocorticotropin hormone (ACTH). Gay and Plant (1987) reported a modest increase in cortisol levels in the rhesus monkey after acute treatment with NMDA. These results were confirmed by Reyes et al. (1990) in long-term ovariectomized monkeys. This group also found that the NMA stimulation of the adrenal axis may account for the inhibitory effect of NMA on LH secretion in their animals. Pretreatment with a corticotropin-releasing factor (CRF) antiserum or the opioid antagonist naloxone, partially prevented the stimulatory effect of NMA on LH secretion (Reyes et al., 1990). The effect of NMDA on the H-P-adrenal axis has also been observed in the rat. Jezova et al. (1991) found that an i.p. injection of either NMDA or NMA caused the dose-related release of ACTH without the increase in epinephrine which is usually associated with a nonspecific stress response. Similarly, Farah et al. (1991) demonstrated that a systemic injection of NMDA rapidly (7 to 15 min.) and transiently (return to basal levels by 60 min.) increased serum ACTH levels. Pretreatment with the NMDA antagonist, CPP, or the glucocorticoid dexamethasone blocked the stimulatory effect of NMDA on ACTH secretion. Recently, Jezova and Oprsalova (1992) have shown that the stimulatory effects on the HPA axis may be partially regulated by the area postrema (AP). The NMDA-evoked increase in ACTH is significantly suppressed in rats with surgical ablation of the AP (Jezova and Oprsalova, 1992). Bardgett et al. (1992) have also reported that neonatal (P9-P10) and juvenile (P20-P21) rats of both sexes increase serum ACTH levels two-fold within 15 min. of NMDA treatment. However, the NMDA antagonist, CPP, did not block the effect in neonatal rats and had only a partial effect in juvenile rats. Recently, Chautard et al. (1993) have reported that NMA, quisqualic acid and kainic acid induce a significant increase in ACTH release of P7 rat pups of both sexes.

Passive immunization with antiserum to CRF blocked NMA-induced ACTH release (Chautard et al., 1993).

Growth Hormone

Glutamate also modulates growth hormone (GH) secretion. Terry et al. (1981) reported that neonatal treatment of rats with neurotoxic doses of MSG which destroyed cells with NMDA receptors severely disrupted GH secretion. Adult rats treated neonatally with MSG had significantly lower levels of circulating GH and lower GH secretory peaks compared to controls (Terry et al., 1981). Nemeroff et al. (1978b) showed that administration of MSG to adult male rats reduced serum GH levels and Terry et al. (1981) demonstrated that MSG given to neonatal rats suppresses the pulsatile pattern of GH release. Mason et al. (1983) however, found that the glutamate agonists NMA and KA increased the serum concentration of GH in adult male rats. Veneroni et al. (1990) reported that in peripubertal rats, MK-801 treatment (10 days; 0.2 mg/kg/day) lowered the pituitary content of GH, the basal GH levels, the growth hormone releasing hormone (GHRH)-stimulateu GH secretion, and the circulating levels of insulin-like growth factor-I (IGF-I). In addition, MK-801 treatment lowered growth rate without impairing food intake. MK-801 treatment regime had no effect on somatostatin-li or somatostatin mRNA in the hypothalamus (Cocilovo et al., 1992).

VI. Thesis Outline

The major consideration of this thesis is the role of the NMDA receptor in the central regulation of sexual maturation in female rats. Our laboratory has previously reported (MacDonald and Wilkinson, 1990) the induction of early puberty through a simple NMDA treatment regime and this thesis will elaborate on this NMDA model of precocious sexual maturation. A relatively minor

portion of this thesis examines a neonatal model of early puberty involving MSG treatment.

Chapter 2 is devoted to the characterization of the NMDA-mediated model of early puberty through measurements of the day of first ovulation, gonadotrophin levels and uterine weight. The remaining chapters in this thesis examine the site of action of NMDA and MSG in the CNS and how they might act to initiate precocious sexual maturation. Specifically, the third chapter examines the expression of an immediate early gene, *c-fos*, in the two models of puberty. The fourth chapter examines the expression of an inducible form of heat shock protein (HSP70) in the NMDA and MSG models of sexual maturation. Finally, the fifth chapter, deals with the possible role of basic fibroblast growth factor (bFGF) in NMDA-initiated early puberty. A short introduction to each of these chapters will precede a description of the experimental results.

CHAPTER 2: GLUTAMATE AND THE REGULATION OF SEXUAL MATURATION

Introduction

Several studies have examined the role of the NMDA receptor in the activation of the reproductive system either at puberty or during the breeding season. In rats and rhesus monkeys, the onset of puberty involves the activation of NMDA receptors. Pulsatile infusion (i.v.) of NMA (Urbanski and Ojeda, 1987) or single daily subcutaneous (s.c.) injections of NMDA (MacDonald and Wilkinson, 1990) accelerate sexual maturation in female rats. Plant et al. (1989) demonstrated that repetitive injections of NMA evokes and maintains pulsatile LH release and results in the premature activation of the reproductive system in male rhesus monkeys. In contrast to NMA, repetitive treatment with L-glutamic acid or kainic acid does not sustain LH release (Medhamurthy et al., 1992). NMDA-treated rats maintain their level of sexual maturity but monkeys revert to their prepubertal state when treatment is discontinued (Urbanski and Ojeda, 1987; Plant et al., 1989). There is also evidence showing that endogenous EAA acting through NMDA receptors regulate sexual maturation. Blockade of NMDA receptors with MK-801 (Veneroni et al., 1990; MacDonald and Wilkinson, 1990; Urbanski and Ojeda, 1990; Meijs-Roelofs et al., 1991) or AP-5 (Wu et al., 1990) delays sexual maturation but does not prevent it. Non-NMDA receptor-mediated neurotransmission is not a critical factor in the process of puberty as kainate and the non-NMDA receptor antagonist, DNQX, did not alter the age or body weight at vaginal opening (VO) compared to control rats (Brann et al., 1993c).

Not only do NMDA receptors play a critical role in sexual maturation but they are also involved in the physiological mechanism that activates and

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deactivates the reproductive axis of seasonal breeding animals. Photoperiodic animals such as hamsters require long days for the initiation and maintenance of reproductive viability. Urbanski (1990) demonstrated that the normal testicular regression associated with short days can be prevented by daily injections of NMDA. In addition, NMDA treatment of short day hamsters with regressed testes increased circulating gonadotrophin and testosterone levels (Urbanski, 1990). Similarly, Colwell et al. (1991) found that MK-801 treatment blocked the stimulatory effect of light on the reproductive axis of short day hamsters and prevented the light-induced reduction in pineal melatonin levels. In contrast to this, Hoover et al. (1992) and Urbanski et al. (1993) showed that NMA treatment did not block the inhibitory effects of melatonin on photo-stimulated hamsters (long day).

In addition, Hsu et al. (1993) have reported that NMDA treatment increases sexual receptivity in female rats as manifested by the post-injection increase in the lordosis quotient. Clearly, EAA and the NMDA receptor are critical components of the neural mechanisms that drive sexual maturation, control the reproductive cycle of photoperiodic animals, and influence sexual behaviour.

Material and Methods

I. Animals

Experimental animals were obtained from Canadian Hybrid Farms (Halls Harbour, N.S.) or Charles River Laboratories (Charles River, Quebec) and maintained during the course of the experiments in the animal quarters (AQ) of the Sir Charles Tupper Medical Building or the Izaak Walton Killam (IWK) Hospital for Children. Upon arrival at the AQ at age P21 or P55, female Sprague-Dawley (SD) rats were placed in plastic cages (4-5 or 2/cage, respectively) and allowed free access to food (Purina Rat Chow) and water. Individual litters of neonatal SD rat pups (P1) were housed with their mother (8-9 pups/dam) in plastic cages until P21 when they were weaned. The day of birth is designated P0. The animals were maintained at a constant temperature (20°C) under a photoperiod of 14:10 hrs. of light:dark (lights on from 0700 to 2100 hr).

II. Drugs

L-Glutamic acid monosodium salt (MSG; G-1626), N-Methyl-D-Aspartic acid (NMDA; M-3262), N-Methyl-D,L-Aspartic acid (NMA; M-2137), luteinizing hormone releasing hormone (LHRH; L-7134) and gonadotropin from pregnant mares' serum (PMSG; G-4877) were obtained commercially from Sigma Chemical Company (St. Louis, MO).

III. 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.).

(1) Collection and Processing of Blood

Rats were quickly decapitated without anaesthesia and trunk blood was collected in 12 x 75 mm glass culture tubes. Blood was stored on ice for at least 2 hrs. (~ 4°C) and then centrifuged at 800 x g for 30 min at 4°C. The resulting serum was transferred to 1.5 ml Eppendorf tubes and stored at -70°C until the LH was quantified by RIA.

(2) Buffers

The LH assay buffer (0.01 M sodium phosphate in 0.15 M sodium chloride) was comprised of the following: 1.0 L saline, 1.0 g gelatin, 0.189 g $NaH_2PO_4 \bullet H_2O$, 1.225 g Na_2HPO_4 and 0.1 g thimerosol. The pH of the buffer solution was adjusted to 7.6 using 4.0 N NaOH. Normal rabbit serum (NRS) buffer 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.

(3) Assay Reagents

The LH standard used was LH RP-3. The first antibody was anti-rat LH S-10. LH I-8 was iodinated (see below) and used as the labeled hormone in the assay. The second or precipitating antibody was Marigold serum and was obtained from Dr. Will Moger, Dept of Physiology and Biophysics, Dalhousie University.

(4) Radioiodination of LH

Twenty µl of LH I-8 was added to a vial of 1 mCi of $Na[^{125}I]$ 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 µl aliquots of each 0.5 ml fraction were counted on a LKB Clinigamma 1272 solid crystal scintillation gamma counter. The fraction showing the best binding as determined 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 antibody) and 0 tubes to determine the amount of specific binding (SB).

(5) Assay Procedure

A series of standards was prepared by serial dilution using the standard LH RP-3. The concentration of these standards ranged from 0 to 10 ng of LH RP-3 per 100 µl. Samples consisted of 100 µl of standard or 50-200 µl of rat serum and were brought to a final volume of 500 µl with LH assay buffer and assayed in duplicate. First antibody (LH S-10) diluted 1:240 with NRS buffer was added in a volume of 200 µl to all tubes except those measuring TC and NSB. Tubes were vortexed and allowed to remain at 25°C for 72 hours. Next, the radioactive label was added in 100µl 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 µl 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 800 x g for 30 minutes at 4°C 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.

(6) 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 log10 of the concentration of the standards is plotted against logit B/B_0. This factor is defined as:

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 0.19 ng of LH and the intra-assay coefficient of variation was 6.0%. The inter-assay coefficient of variation was 10.5%. The serum pool was obtained from ovariectomized adult female rats (6 weeks post-surgery) and was assayed at two dilutions (x1 and x5). All samples from each experiment were assayed together. Values are expressed as means ± s.e.m. and in terms of ng rat LH RP-3/ml of serum.

IV. VO experiments

In our colony VO occurs at approximately postnatal day 33 (P33) and is coincident with first ovulation in >90% of rats. The remaining rats are usually in a state of proestrus (bloated, fluid-filled uteri).

The glutamate agonists, NMDA and NMA have been used interchangeably in the following experiments. The more expensive drug, NMDA ($258.55 U.S./g; 20 mg/kg) was used in the initial experiments. Later, the racemic form of the drug was used, NMA ($59.70 U.S./g; 30 mg/kg), after it was determined that the less expensive drug was equally effective in inducing precocious puberty and releasing LH as NMDA (see VO experiments 5, 6 and LH experiment 1).

(1) Effect of NMDA on Sexual Maturation

Previous work has shown that daily injections of NMDA (15 mg/kg) from the age of 27 days to VO synchronize and slightly accelerate sexual maturation (MacDonald and Wilkinson, 1990). In this experiment, the effects of a higher dose of NMDA (20 mg/kg) initiated earlier in development (P24) were investigated. NMDA injections (20 mg/kg; s.c.; n=10) were administered daily at 1100 h and control rats were treated with 0.9% saline (0.1 ml; s.c.; n=10) from P24 to VO. The rats were weighed daily and monitored for VO.

(2) The Effect of NMDA on VO when Injections are Initiated Close to Puberty

The ability of NMDA to accelerate and synchronize sexual maturation was evaluated when NMDA treatment was initiated close to unstimulated puberty. In our colony, the earliest age at which control rats reach VO is P30. Juvenile female rats (n=10/group) were given daily injections (s.c.) of NMDA (20 mg/kg) at 1100 h beginning at 24 or 29 days of age until VO was observed. Control rats

were injected (s.c.) with saline (0.1 ml) beginning on P24. On the day of VO, each animal was weighed, sacrificed and the ovaries examined for corpora lutea (CL).

(3) The Effect of a High and a Low Dose of NMDA on the Onset of Puberty

We examined the sensitivity of hypothalamic glutamate receptors by comparing the puberty-inducing effects of a high (20 mg/kg) and a low (5 mg/kg) dose of NMDA. The rats were weighed and injected (s.c.) at 1100 h each day with either 5 or 20 mg/kg of NMDA from P24 to the day VO was observed (n=10/group). Control rats were injected (s.c.) with 0.9% saline (0.1 ml; n=10) using the same protocol as the NMDA groups. The estrus cycle of each rat was monitored using daily vaginal smears for 12 to 14 days after they reached VO. The vaginal smears were taken by saline lavage between 1300 and 1400 h each day.

(4) The Effect of Injection Time on NMDA-Induced Precocious Puberty

To determine whether the stimulatory effect of NMDA on sexual maturation was linked to the time of injection, groups of rats (n=10/group) were administered NMDA (20 mg/kg; s.c.) at different time points during the day and monitored for VO. Daily injections of NMDA were made at either 1100 h, 1400 h or 1800 h beginning on P24. Control rats were injected with 0.9% saline (0.1 ml; s.c.) at 1100 h and handled at 1400 and 1800 h. Body weights were measured daily and treatment was continued until VO was observed. On the day of VO, the rats were sacrificed and the ovaries were inspected for the presence of CL.

(5) A Comparison of the Effects of NMA and NMDA on Sexual Maturation

Here, we investigated whether the racemic mixture NMA was as effective as NMDA at inducing precocious VO. Female rats were injected (s.c.) daily with NMDA (20 mg/kg; n=20) or NMA (20 or 30 mg/kg; n=10/dose) from P24 until

VO was observed. Injections were given at 1100 h each day and control rats were injected with 0.9% saline (0.1 ml; n=10). On the day of first ovulation, the rats were sacrificed and the ovaries were inspected for the presence of CL.

(6) The Effect of Age of Initial NMA Injection on Sexual Maturation

We examined the ability of various NMA injection schedules to promote precocious sexual maturation. The purpose of this study was to evaluate the relative importance of two treatment parameters (age of initial injection and number of days of treatment) in the initiation of sexual development. Immature female rats (n=6-36/group) were injected (s.c.) daily at 1100 h with NMA (30 mg/kg) using one of 8 injection schedules: P24 to VO; P24 to P28; P24 to P27; P25 to P28; P21 to VO; P21 to P28; P21 to P25; or P16 to P20 (pre-weanling). Control rats were injected (s.c.) with saline (0.1 ml) from P24 to VO.

(7) The Effect of 2 or 3 Daily Injections of NMA on the Onset of Puberty

This experiment was divided into two sections. In the first part of the experiment, the effect of doubling the dosage of NMA on sexual maturation was investigated by injecting (s.c.) twice a day with NMA (30 mg/kg). Juvenile female rats were administered NMA at either 1300 h (n=10; NMA x1) or at 1300 h and 1700 h (n=10; NMA x2) from P24 to P28. Control rats (n=9) were injected (s.c.) twice daily at 1300 and 1700 h with 0.9% saline (0.1 ml). In the second half of the experiment, the effect of tripling the dosage of NMA on the onset of puberty was investigated by injecting three times a day with NMA (30 mg/kg). Juvenile female rats were administered NMA at either 1300 h (n=10; NMA x1) or at 1300, 1500 and 1700 h (n=10; NMA x3) from P24 to P28. Another group of rats (n=10) were injected three times each day at 1300, 1500 and 1700 h with 0.9% saline to control for the stress of multiple injections. After the injections were

discontinued the rats were monitored daily for VO, the day of first ovulation and body weight for each rat was recorded.

(8) The Effect of NMDA Treatment on Uterine Weight in Female Rats

To determine the effect of NMDA injections on uterine weight (estrogen-dependent) groups of animals were killed at different time points during and after treatment. Immature female rats were treated with NMDA (20 mg/kg; s.c.) or saline (0.1 ml; s.c.) at 1100 h each day from P24 until P28. On P24 (n=15/group), P26 (n=15-18/group), P28 (n=38-48/group) and P30 (n=8-10/group), rats from both the treated and control groups were sacrificed. The uteri were removed, trimmed of fat and weighed. The results are a composite of three separate experiments using the identical protocol described above.

(9) The Effect of NMA or PMSG Treatment on Uterine Weight in Pre-Weanling Rats

The purpose of this experiment was to investigate whether the pituitary-gonadal axis of pre-weanling rats can respond to enhanced gonadotrophic drive with increased estrogen production. Rats were injected with PMSG (20 IU; s.c.; n=13) at 1600 h on P16. Two other groups received saline (0.1 ml; s.c.; n=13) or NMA (30 mg/kg; s.c.; n=14) daily at 1600 h from P16 to 20. At 21 days of age, the rats were weighed, checked for VO and sacrificed rapidly by decapitation. Uteri were removed, trimmed of fat and weighed. The ovaries and oviducts were checked for the presence of CL and oocytes, respectively.

(10) The Effect of NMDA Treatment (P21 to P25) on Uterine Weight

Young female rats were injected with NMDA (20 mg/kg; s.c.) or saline daily for 5 days during the earliest period where NMDA treatment can induce precocious puberty (P21 to 25). At 26 days of age, 10 rats from the control and the treated group were sacrificed and uteri were removed, trimmed of fat and

weighed. The rats were monitored for VO between 26 and 29 days of age. On P29, the remaining animals in the NMDA (n=13) and the control (n=11) group were sacrificed and the uterine weight measured.

(11) The Effect of Daily LHRH Injections on VO and Uterine Weight

We also investigated whether stimulation of the pituitary-ovarian axis with daily injections of LHRH, at a dose which gives a peak LH response in the same range as NMDA (20 mg/kg; see Results for LH Experiments 4 and 5) could accelerate sexual maturation and increase estrogen production. Juvenile female rats were administered NMDA (20 mg/kg; s.c.; n=21) or LHRH (5 ng/100g; s.c.; n=21) or saline (0.1 ml; s.c.; n=20) daily from P24 to P28. Half of each group (n=10/group) were weighed and monitored for VO daily after the injections were discontinued on P28. The other half of each treatment group were weighed, checked for VO and sacrificed rapidly by decapitation on P29. Uteri were removed, trimmed of fat and weighed.

(12) The Effect of Bromocriptine on NMDA-Induced Early Puberty

The purpose of this experiment was to investigate the possible role of prolactin in NMDA-induced precocious puberty. Juvenile female rats were injected (s.c.) with a suspension of bromocriptine methanesulfonate (0.5 mg/rat), a pituitary dopamine receptor agonist, or saline vehicle between 1000 and 1100 h from P24 to P28. In the afternoon (1600 h) of this treatment period, half of the bromocriptine group and half of the control group were injected (s.c.) daily with either NMDA (20 mg/kg) or saline. The four groups: bromocriptine/NMDA (n=10), bromocriptine/saline (n=10), saline/NMDA (n=10), saline/saline (n=10) were monitored for VO in the afternoon after the injections were discontinued. On the day of VO, the rats were sacrificed and their ovaries were inspected for the presence of CL.

(13) The Combined Effect of Neonatal MSG and Prepubertal NMDA on Sexual Maturation

In this experiment, the effect of neonatal MSG treatment (known to induce precocious puberty; MacDonald & Wilkinson, 1990) on NMDA-induced precocious puberty in female rats was examined. Eight litters of 8-9 female pups were treated with MSG (2 mg/g; s.c.) or 0.9% saline (0.1 ml; s.c.) on P2. The rats were weighed and weaned on P21 and animals that weighed 54.0 ± 5 g were included in the second half of this study (n=56). Beginning on P24, the MSG treated rats and the control rats were injected with NMDA (20 mg/kg; s.c.) or saline (0.1 ml; s.c.) and the treatment was continued until VO was observed. At VO, the rats in the 4 groups: MSG/NMDA, MSG/saline, saline/NMDA, saline/saline (n=14/group), were sacrificed and their ovaries and oviducts were examined for the presence of CL and oocytes.

V. LH Experiments

(1) The Effect of Different NMA Doses on LH secretion

The effect of different doses of NMA on LH secretion was examined. On the morning of P28, immature female rats (n=10/dose) were injected (s.c.) with NMA (5, 10, 20, 30 mg/kg) or saline (0.1 ml). They were sacrificed rapidly by decapitation 8 min. post-injection (peak LH response; MacDonald and Wilkinson, 1992) and trunk blood was collected.

(2) Alterations in the LH Response to NMDA During NMDA-Induced Puberty

The LH response to NMDA may change during the series of daily injections used to initiate precocious VO. To investigate this hypothesis we injected (s.c.) groups of rats with either NMDA (20 mg/kg) or saline from P24 to VO. The LH response to NMDA was measured in rats which were receiving NMDA for the first time (previous saline injections; first NMDA inj'n) and in rats which had received previous daily injections of NMDA (multiple NMDA inj'ns). Rats from both groups were sacrificed rapidly by decapitation 8 minutes after injection (s.c.) of NMDA (20 mg/kg) on P24, P26, P28, P30, P32 and on the day of first ovulation (variety of days) and trunk blood was collected.

(3) LH Dose Response to NMDA on P28 in Control and NMDA-Treated Rats

The data from Experiment LH2 showed that the largest change in LH response occurs on day 28 (immediately after the fifth injection). To further characterize this change, the LH response to various doses of NMDA was measured in control and treated (20 mg/kg NMDA/day from P24-27) rats on day P28. Immature female rats which had received daily injections of saline or NMDA (20 mg/kg) from day P24 were injected with NMDA (2.5, 5, 10, 20

mg/kg). Animals were killed 8 min post-injection and trunk blood was collected.

(4) LH Dose Response to LHRH on P28 in Control and NMDA-Treated Rats

To determine whether the decline in NMDA-induced LH secretion following daily injections of NMDA occurs due to a decrease in pituitary (LHRH) or hypothalamic (NMDA) responsiveness, the LH response to various doses of LHRH was measured in control and treated (20 mg/kg NMDA from P24-P27) rats. Immature female rats which had received daily injections of saline or NMDA (20 mg/kg) from P24 to P28 were injected with LHRH (2.5, 10, 20, and 50 ng/100g) on P28. Animals were killed 15 min post-injection and trunk blood was collected.

(5) The Time Course of the LH Response to NMA and LHRH after Previous NMA and LHRH Treatment

The purpose of this experiment was to determine if a series of daily injections of NMA or LHRH changes the time course of LH release. Female rats were injected (s.c.) with NMA (30 mg/kg) or LHRH (5 ng/100g) or 0.9% saline (0.1 ml) daily at approximately 1000 h from P24 to P27. Rats that had been pre-treated with 4 daily injections of NMA or LHRH were injected for the 5th time on P28 and at several time points after injection (t=0, 8, 15, 30 and 60 min.; n=9-10/time pt.) they were sacrificed by decapitation and trunk blood was collected. The rats that had received saline injections from P24 to P27 were injected with NMA (30 mg/kg; s.c.; n=49) or LHRH (5 ng/100g; s.c.; n=49) at 28 days of age and at several time points post-injection (n=9-10/time pt.) they were sacrificed by decapitation and trunk blood was collected.

VI. Statistics

Statistical analyses of data were performed on an Apple Macintosh IIci using Statview II or IV (Abacus Concepts). Determination of significant treatment effects was accomplished using either one factor or two factor analysis of variance (ANOVA). Significant differences between group means were analyzed by *post hoc* Dunnett t-tests or Student t-tests with $p<0.05$ denoting a significant difference.

Results

I. VO experiments

(1) Effect of NMDA on Sexual Maturation

The results of this experiment were combined and plotted with the results for experiment 2 in MacDonald and Wilkinson (1990) because the mean day of VO for control rats was very similar in both experiments (control from P27 experiment: 33.8 ± 0.3 days; control from P24 experiment: 33.2 ± 0.8). As illustrated in Figure 3, treatment with a higher dose of NMDA (20 mg/kg/day) initiated earlier (P24) is much more effective at advancing the day of VO than treatment with a lower dose (15 mg/kg/day) initiated later in development (NMDA, P27, 15 mg/kg: 32.5±0.2 days (n=10); NMDA, P24, 20 mg/kg: 29.5±0.5 days (n=10); Control: 33.3±0.4 days (n=10 + 11 = 21)). The P27 NMDA group is slightly but not significantly precocious compared with the controls. The P24 NMDA group reach VO significantly (p<0.005) sooner than both the control and the P27 NMDA group. In contrast, synchronization of the day of VO is not as prominent in the d24 NMDA group as it is in the d27 NMDA group (Fig. 4). Note that the P27 group reach VO over the course of 2 days whereas the P24 and control group show VO over 6 and 8 days, respectively.

The mean body weight at VO was 109.5 ± 1.1 g and 119.9± 3.5 g for the two control groups. The rats treated with NMDA from P27 and P24 reach VO at a mean body weight of 101.5 ± 1.0 g and 105.0 g ± 3.0 g which is 8 and 15 g lower than controls, respectively.
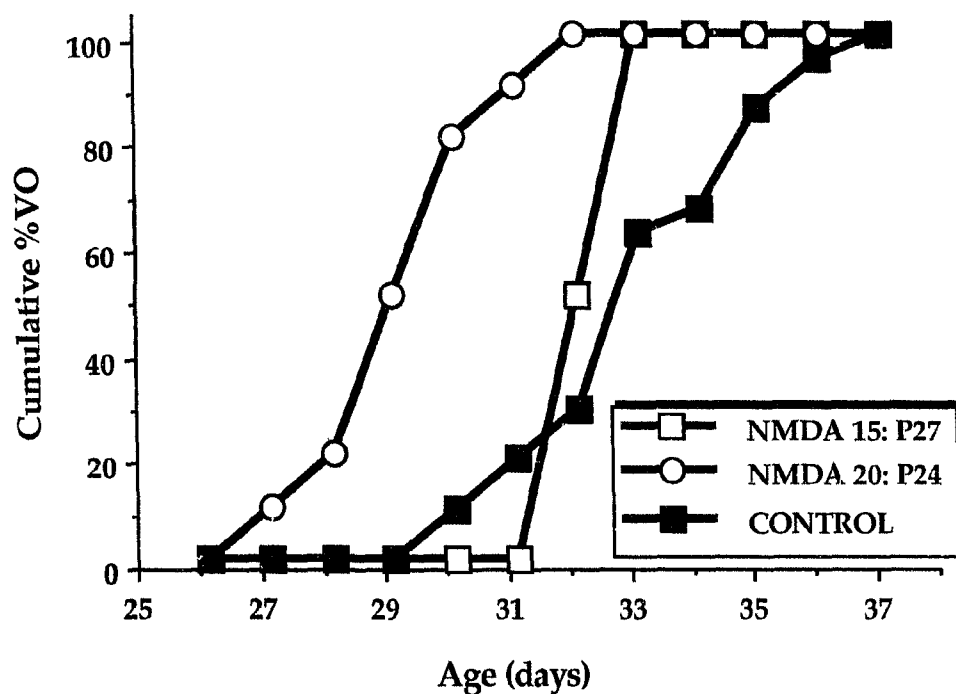
Figure 3. Effect of two prepubertal NMDA treatments on the timing of VO (Cumulative %VO).

The treatments were: (1) 15 mg/kg NMDA (s.c.) daily (1100 h) from P27 to the day of VO (n=10; Mike MacDonald's thesis); and (2) 20 mg/kg/day NMDA (s.c.) -- 1100 h from P24 to the day of VO (n=10). Control rats (n=21; 10 from Mike MacDonald's thesis) received daily (1100 h) injections of saline (s.c., 0.1 ml) from P24 to VO. Data are plotted as the cumulative percentage of rats showing VO at each age.
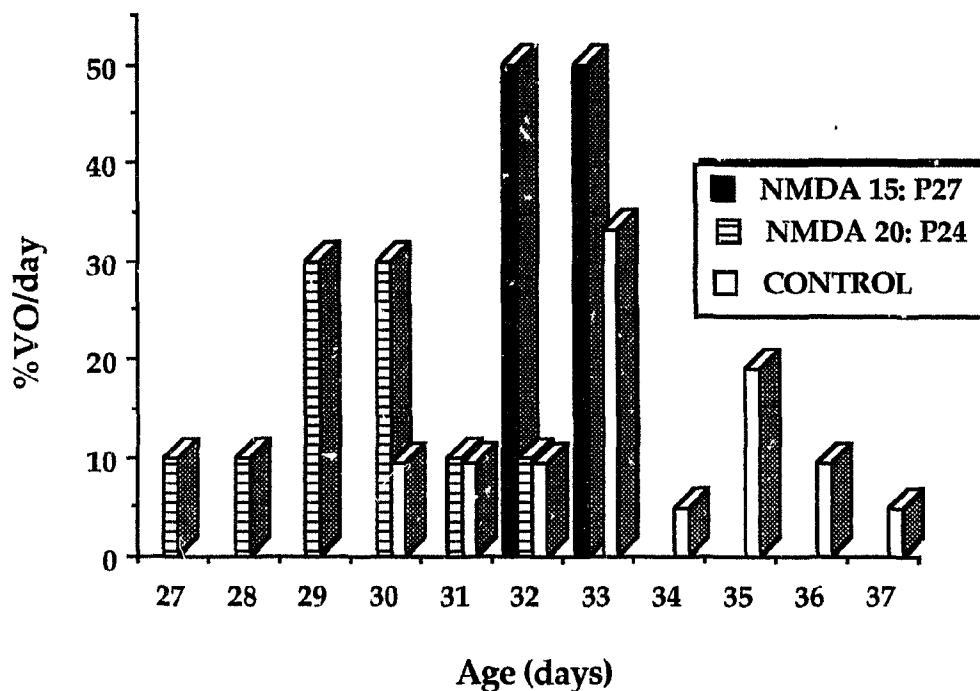
Figure 4. Effect of two prepubertal NMDA treatments on the timing of VO (%VO/day).

(1) 15 mg/kg NMDA (s.c.) daily (1100 h) from P27 to the day of VO (n=10); (2) 20 mg/kg/day NMDA (s.c.) at 1100 h from P24 to the day of VO (n=10). Control rats (n=21) received daily (1100 h) injections of saline (s.c., 0.1 ml) from P24 to VO. Data are plotted as the percentage of rats showing VO at each age.

(2) The Effect of NMDA on VO when Injections are Initiated Close to Puberty

The cumulative %VO of female rats which were treated with 0.9% saline or with NMDA (20 mg/kg) from P24 or P29 are shown in Fig. 5. NMDA injections initiated close to puberty (P29) are capable of slightly but significantly ($p<0.05$) accelerating the mean day of first ovulation (NMDA P24: 30.5 ± 0.4 days (n=10); NMDA P29: 32.3 ± 0.2 (n=10); Control: 34.0 ± 0.9 (n=10). However, the body weight (g) at first ovulation in the P29 group is lower but not significantly different from the control group (NMDA P24: 106.2 ± 2.3; NMDA P29: 120.2 ± 1.4; Control: 127.1 ± 4.4). In agreement with Experiment 1, NMDA treatment initiated close to unstimulated puberty (from P27 or P29) synchronizes the day of first ovulation (Fig. 6). In contrast, treatment initiated on P24 induces a large precocity (4 days) with a small effect on synchronization. The mean number of CL per rat at first ovulation was not significantly different from that in controls (NMDA P24: 13.3 ± 0.7; NMDA P29: 12.8 ± 0.3; Control: 12.0 ± 0.9).

(3). The Effect of a High and a Low Dose of NMDA on the Onset of Puberty

A high dose (20 mg/kg) but not a low dose of NMDA (5 mg/kg) significantly ($p<0.005$) advances the mean day of VO (NMDA 5 mg/kg: 32.7 ± 0.4 days (10); NMDA 20 mg/kg: 30.0 ± 0.3 (10); Control: 33.5 ± 0.5 (10)) (Fig. 7). However, the mean body weight at first ovulation of both treated groups is significantly lower than the controls (NMDA 20: $97.7^{**}\pm2.6$ g; NMDA 5: $113.2^{*}\pm3.0$ g; Control: 120.2 ± 2.6 g; $^{*}p<0.05$ and $^{**}p<0.005$ compared to control). Post-pubertal control and NMDA-treated rats exhibited regular 4-5 day estrous cycles over a two week period of study (Fig. 8).
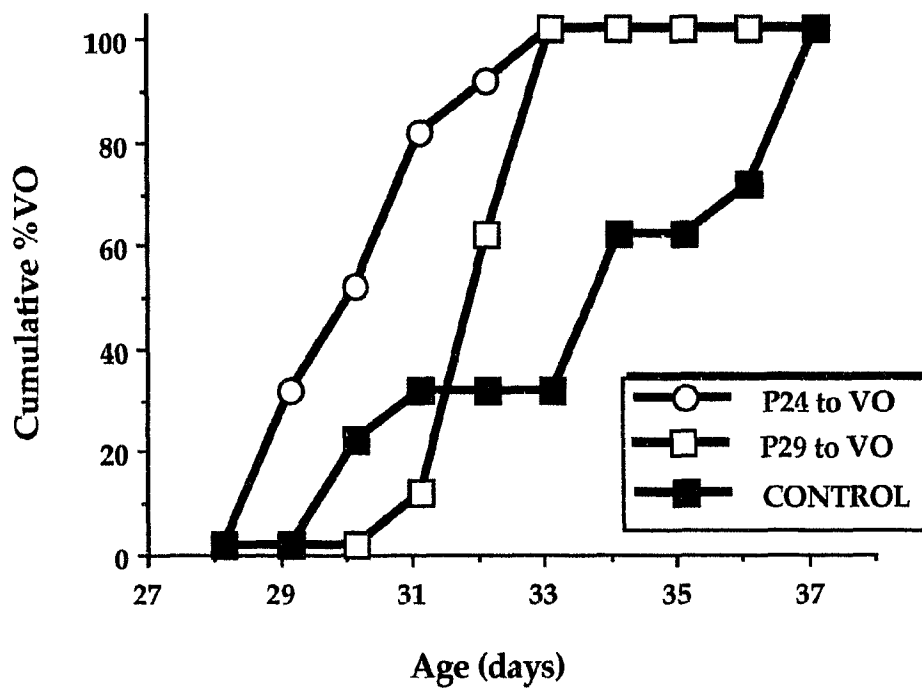
Figure 5. The effect of NMDA treatment from P24 and P29 on the timing of VO (Cumulative %VO).

Effect of daily (1100 h) NMDA (20 mg/kg) injections (s.c.) from P24 (n=10) or P29 (n=10) to VO on the timing of sexual maturation. Control rats (n=10) received daily (1100 h) injections of saline (s.c., 0.1 ml) from P24 to VO. Data are plotted as the cumulative percentage of rats showing VO at each age.
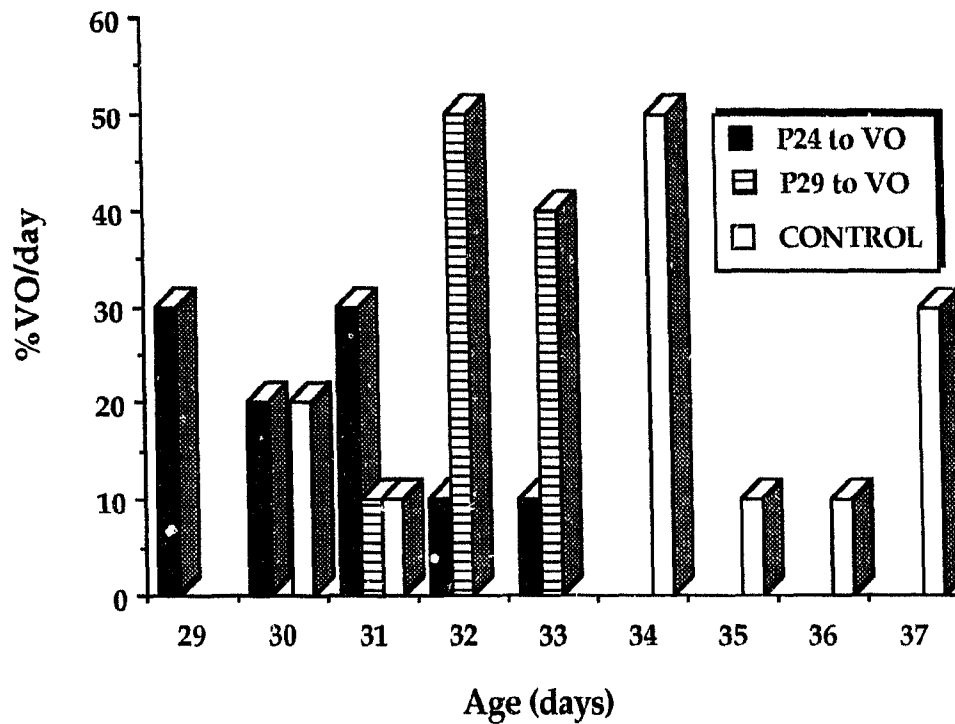
Figure 6. The effect of NMDA treatment from P24 and P29 on the timing of VO (%VO/day).

Effect of daily (1100 h) NMDA (20 mg/kg) injections (s.c.) from P24 (n=10) or P29 (n=10) to VO on the timing of sexual maturation. Control rats (n=10) received daily (1100 h) injections of saline (s.c., 0.1 ml) from P24 to VO. Data are plotted as the percentage of rats reaching VO at each age.
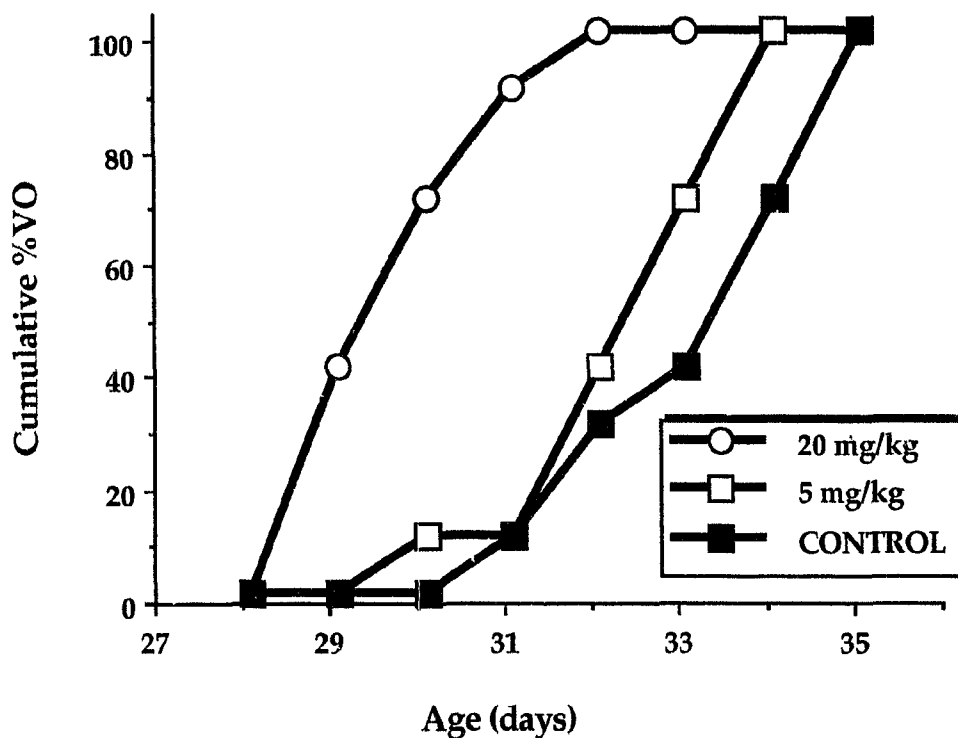
Figure 7. The effect of different doses of NMDA on the onset of puberty.

Effect of 5 mg/kg (n=10) or 20 mg/kg (n=10) of NMDA administered (s.c.) daily (1100 h) from P24 to VO on the onset of puberty. Control rats (n=10) received daily (1100 h) injections of 0.9% saline (s.c., 0.1 ml) from P24 to VO. Data are plotted as the cumulative percentage of rats showing VO at each age.
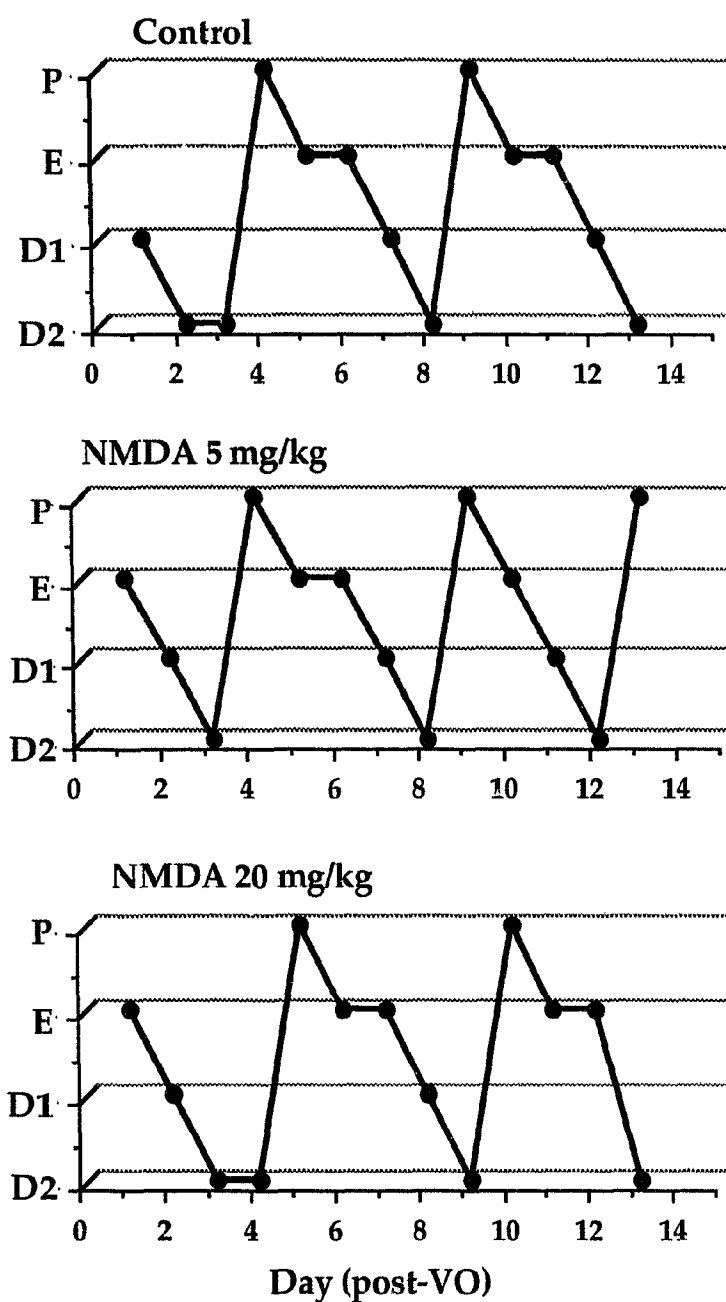
Figure 8. The Effect of NMDA treatment on post-pubertal estrous cyclicity.
Effect of 5 mg/kg (n=10) or 20 mg/kg (n=10) of NMDA administered (s.c.) daily (1100 h) from P24 to VO on post-pubertal estrous cyclicity. The top graph is representative of the estrous cycles of saline-treated rats and the middle and bottom graph are representative of the estrous cycles of NMDA (5 and 20 mg/kg) treated rats. P - proestrus; E - estrus; D1 - diestrus 1 or metaestrus; D2 - diestrus 2.

(4) The Effect of Injection Time on NMDA-Induced Precocious Puberty

NMDA treatment had the same stimulatory effect on sexual maturation when administered at various times during the day. All NMDA-treated groups reached VO significantly (p<0.005) earlier than the controls (NMDA 1100 h: 30.5 ± 0.3 days (n=11); NMDA 1400 h: 30.4 ± 0.3 (n=11); NMDA 1800 h: 30.5 ± 0.4 days (n=10); Control: 33.6 ± 0.5 days (n=19)) (Fig. 9). In addition, all treated groups reached puberty at a significantly (p<0.005) lower body weight than controls (NMDA 1100 h: 104.9 ± 2.3 g; NMDA 1400 h: 106.5 ± 2.3; NMDA 1800 h: 106.2 ± 2.3; Control: 125.9 ± 1.2). There was no difference in the mean day of VO or body weight at VO between NMDA-treated groups. The mean number of CL per rat at first ovulation in the NMDA-treated groups was not significantly different when compared to controls (NMDA 1100 h: 13.2 ± 0.6; NMDA 1400 h: 13.1 ± 0.6; NMDA 1800 h: 13.4 ± 0.8; Control: 13.0 ± 0.6).

(5) A Comparison of the Effects of NMA and NMDA on Sexual Maturation

Figure 10 shows that the racemic mixture, N-methyl-D,L-aspartic acid (NMA) can induce precocious sexual maturation as effectively as daily NMDA injections. NMDA (20 mg/kg) and both doses of NMA (20 and 30 mg/kg) significantly (p<0.005) lower the mean day of VO and the body weight at VO. (NMDA 20: 30.9±.3 days, 101.4±2.1 g (n=20); NMA 20: 31.3±0.4 days, 105.2±2.9 g (n=10); NMA 30: 31.1±.5 days, 106.2±3.5 g (n=10); Control: 34.1±.7 days, 121.3±4.6 g (n=10). In contrast, the mean CL per animal on the day of VO was very similar between treatment groups and controls (NMDA 20: 13.4 ± 0.3; NMA 20: 13.6 ± 0.5; NMA 30: 13.5 ± 0.6; Control: 13.3 ± 0.5).
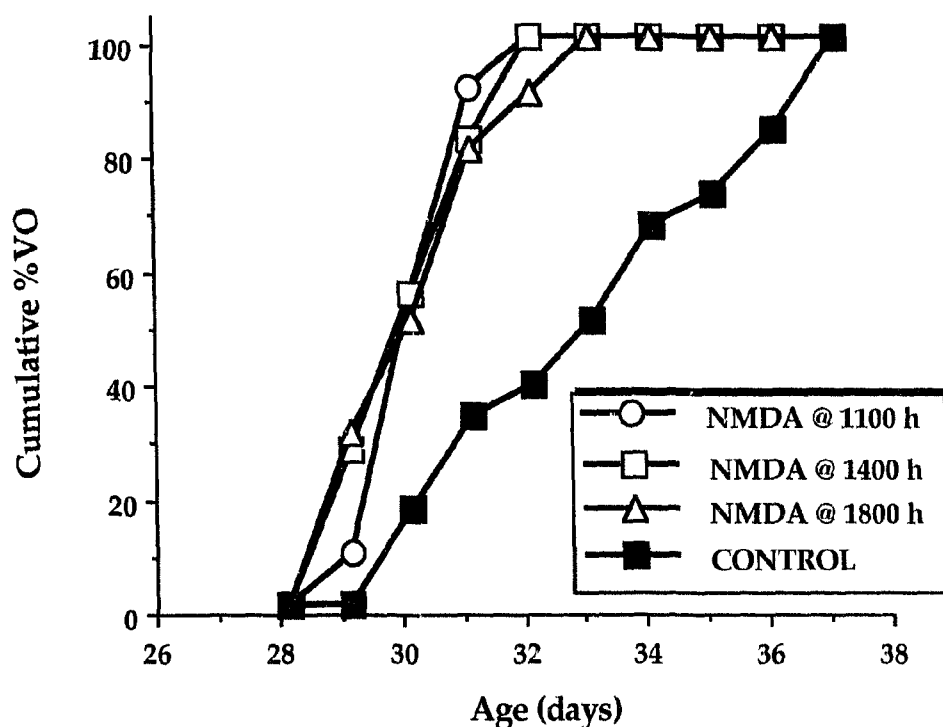
Figure 9. The effect of time of injection of NMDA on the onset of puberty.

Effect of injection time on the stimulatory effects of daily injections of NMDA (20 mg/kg) from P24 to VO on the timing of VO. (1100 h (n=11); 1400 h (n=11); 1800 h (n=10)). Control rats (n=18) received daily (1100 h) injections (s.c., 0.1 ml) of saline and were handled at 1400 h and 1800 h from P24 to VO. Data are plotted as the cumulative percentage of rats showing VO at each age.
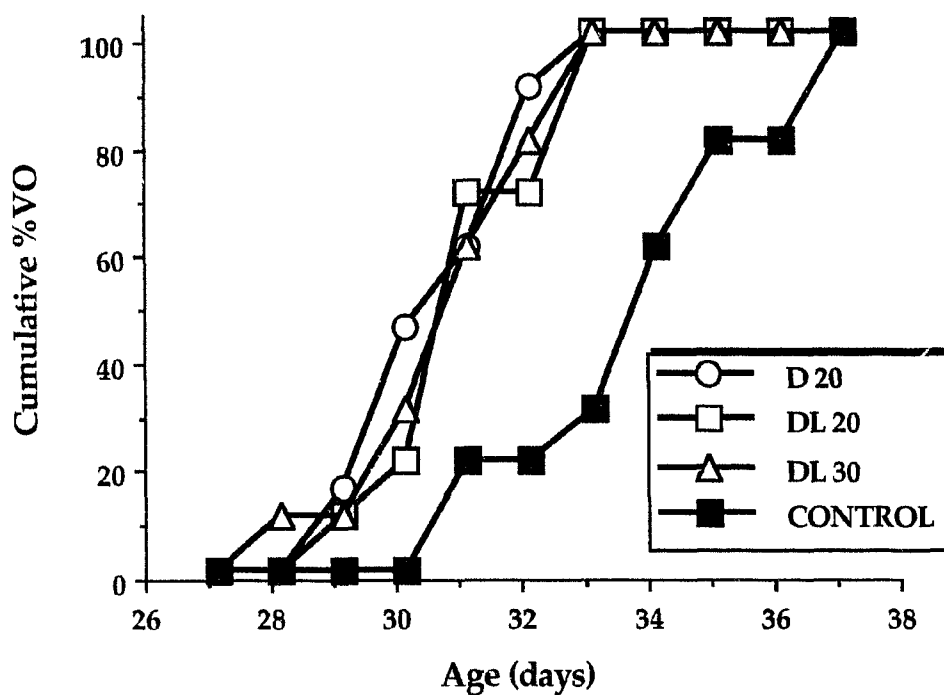
Figure 10. A comparison of the effect of NMA and NMDA on the onset of puberty.

The effect of the D (20 mg/kg; n=20) and the D,L (20 and 30 mg/kg; n=10/dose) form of NMA administered (s.c.) daily (1100 h) from P24 to VO on the onset of puberty. Control rats (n=10) received daily (1100 h) injections (s.c., 0.1 ml) of saline. Data are plotted as the cumulative percentage of rats showing VO at each age.

(6) The Effect of Age of Initial NMA Injection on Sexual Maturation

The effect of various NMA injection schedules on the mean day of first ovulation and mean body weight at VO are summarized in Table 2. With the exception of the P16 to P20 group, all of the NMA injection schedules significantly advance sexual maturation and lower mean body weight at first ovulation. Note that five daily injections of NMA administered as early as P21 to 25 induces the same precocity as the P21 to VO group.

(7) The Effect of 2 or 3 Daily Injections of NMA on the Onset of Puberty

The results from both sections of this experiment have been pooled because the mean day of VO and the mean weight at first ovulation are virtually identical for the control groups and the NMA (x1) treated groups. As illustrated in Fig. 11, two or three daily injections of NMA (30 mg/kg/injection) significantly advance VO but are not more effective than one daily injection of NMA (30 mg/kg/day) (NMA x1: 30.3 ± 0.2 days; NMA x2: 30.2 ± 0.5; NMA x3: 30.0 ± 0.6; Control: 32.9 ± 0.4; $p < 0.005$). In addition the mean body weight at VO of all three treated groups is significantly lower than the control group but there was no difference in mean body weight at VO between treated groups (NMA x1: 95.3 ± 1.6 g; NMA x2: 94.3 ± 2.9; NMA x3: 96.5 ± 2.7; Control: 107.7 ± 2.0).

Table 2. The effect of different NMA injection schedules on the mean day of first ovulation and the mean body weight at first ovulation.

	n	Mean Age at VO (days)	Mean Wt at VO (g)
Control	10	33.7 ± 0.6	108.5 ± 2.8
Day 24 to VO	20	29.9 ± 0.3*†	90.6 ± 1.7*†
Day 24 to 28	36	29.9 ± 0.3*†	90.7 ± 1.4*†
Day 24 to 27	10	30.3 ± 0.5*†	93.4 ± 3.2*†
Day 25 to 28	10	30.6 ± 0.6*†	92.8 ± 2.5*†
Day 21 to VO	10	30.4 ± 0.4*†	93.8 ± 2.2*†
Day 21 to 28	10	29.0 ± 0.5*†	89.7 ± 2.4*†
Day 21 to 25	10	29.8 ± 0.9*†	92.4 ± 4.8*†
Day 16 to 20	6	33.5 ± 0.6	106.2 ± 6.9

Immature female rats were injected (s.c.) daily with NMA (30 mg/kg) using one of 8 injection schedules: P24 to VO; P24 to 28; P24 to 27; P25 to 28; P21 to VO; P21 to 28; P21 to 25; or P16 to 20 (pre-weanling). Control rats were injected (s.c.; 0.1 ml) with saline from P24 to VO. One factor ANOVA and *post hoc* Dunnett's t-tests. *$p<0.05$ compared with P16 to 20; †$p<0.05$ compared with controls. Values are group means $\pm$ s.e.m.
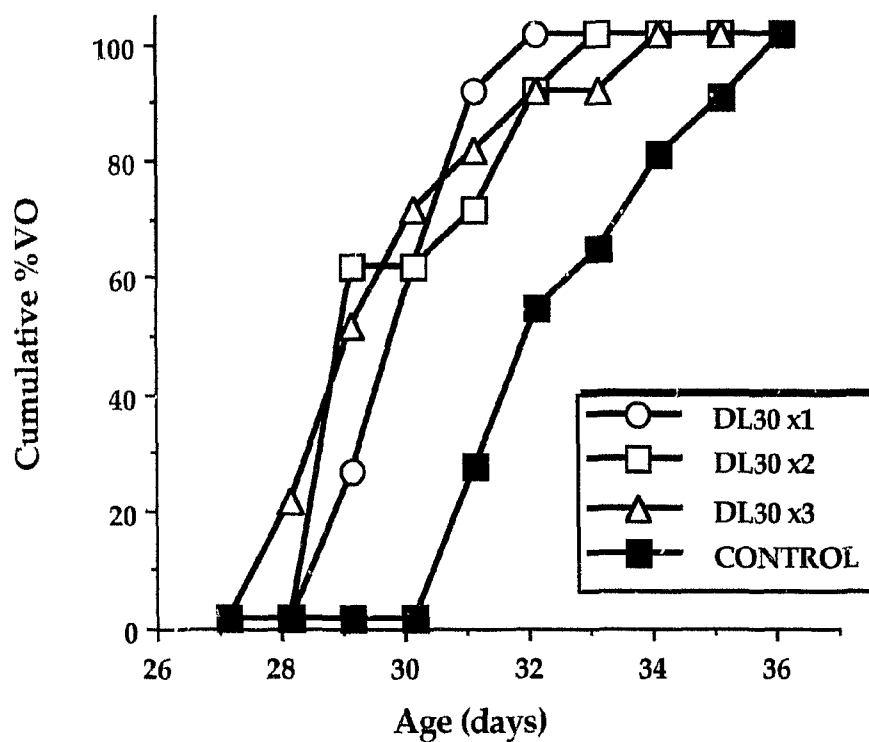
Figure 11. The effect of one, two or three daily injections of NMA on the onset of puberty.

The effect of one (30 mg/kg @ 1100 h; n=10) vs. two (30 mg/kg @ 1300h & 1700 h; n=10) vs. three (30 mg/kg @ 1300, 1500 & 1700 h; n=10) daily injections of NMA from P24 to 28 on the timing of VO. Control rats (n=19) received two or three daily injections (s.c.) of saline (0.1 ml) from P24 to 28. Data are plotted as the cumulative percentage of rats showing VO at each age.

(8) The Effect of NMDA Treatment on Uterine Weight in Female Rats

Uterine weight is estrogen-dependent and is a reliable bioassay for serum estradiol levels. Daily NMDA injections from P24 to 28 induce a progressive rise in uterine weight (Fig. 12). A 2-way ANOVA revealed significant main effects of treatment ($F_{1,159} = 83.5$; $p<0.001$) and age ($F_{3,159} = 77.4$; $p<0.001$) and a significant treatment by age interaction ($F_{3,159} = 21.2$; $p<0.001$). Post hoc Student's t-tests revealed that on P26, P28 and P30, the uteri of prepubertal (nonVO) NMDA-treated female rats were significantly ($p<0.05$ for P26; $p<0.005$ for P28 and P30) larger than the uteri of age-matched (nonVO) control females.

(9) The Effect of NMA or PMSG Treatment on Uterine Weight in Pre-Weanling Rats

PMSG (P16; 20 IU), but not NMA treatment (P16-20; 30 mg/kg/day; s.c.), had a significant stimulatory effect on the pituitary-gonadal axis of P21 female rats. At 21 days of age, 7 of 13 rats in the PMSG group had reached VO and when the uteri were examined another 2 rats had bloated, fluid-filled uteri. Post hoc Dunnett t-tests revealed that the mean uterine weight of the PMSG group was significantly ($p<0.005$) larger than the NMDA or the control group (PMSG: 126.1 ± 4.9 mg (n=13); NMDA: 29.0 ± 1.2 (n=14); control: 26.7 ± 0.6 (n=13)) (Fig. 13). Although PMSG treatment had a stimulatory effect on uterine weight, none of the rats had reached first ovulation when the ovaries and oviducts were examined for the presence of CL and oocytes.
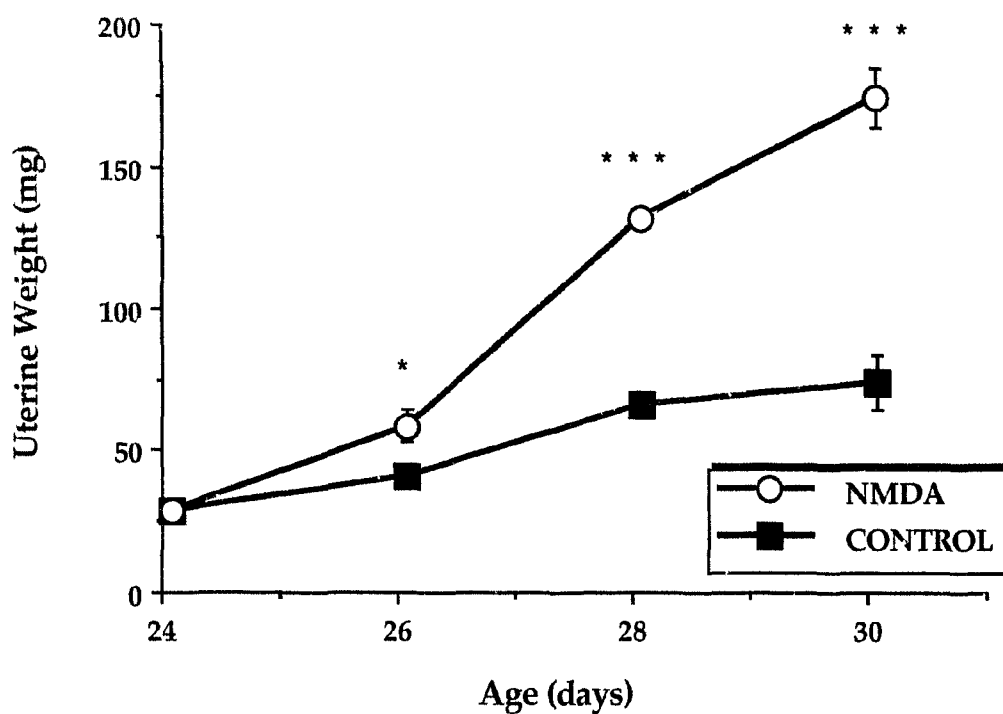
Figure 12. The effect of NMDA treatment on uterine weight.

Effect of daily (1100 h) injections (s.c.) of NMDA (20 mg/kg; P24 to 28) on uterine weight in nonVO animals between 24 and 30 days of age. Control rats received daily (1100 h) injections (s.c.) of 0.9% saline (0.1 ml) from P24 to 28. Subgroups of the controls and treated were sacrificed on P24 (n=15), 26 (con: n=15; NMDA n=18), 28 (con: n=38; NMDA: n=48) and 30 (con: n=8; NMDA: n=10). Values are group means ± s.e.m. *p<0.05 and ***p<0.005 compared with the age-matched control group; 2 factor ANOVA and *post hoc* Student's t-test.
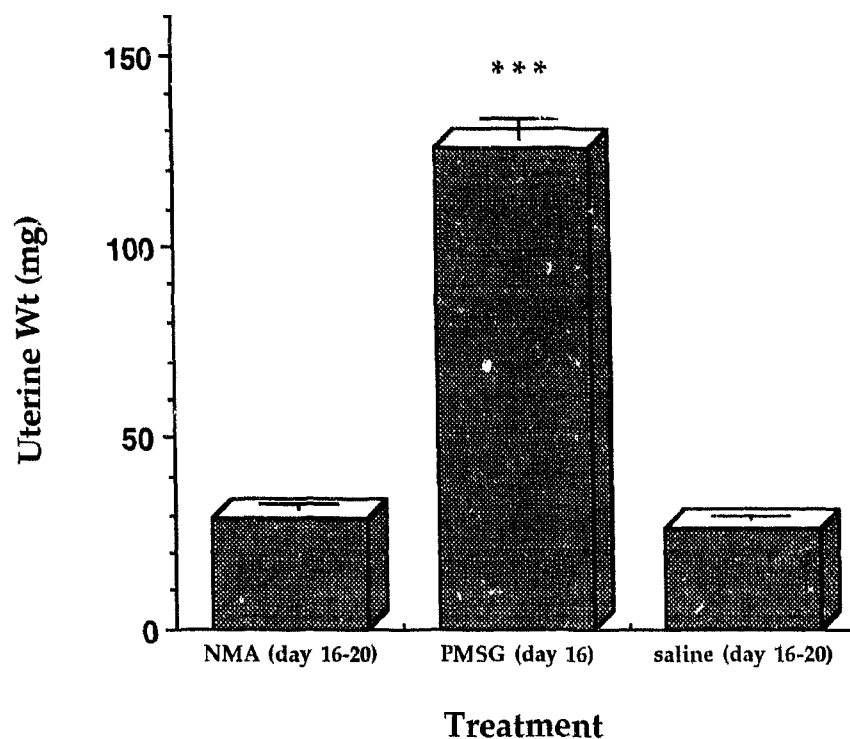
Figure 13. The effect of NMA or PMSG on uterine weight of P21 female rats.

Effect of PMSG (20 IU on P16; s.c.; n=13) or NMA (30 mg/kg/day; P16 to 20; s.c.; n=14) on the uterine weight of P21 female rats. Control rats (n=13) received daily injections of saline at 1600 h from P16 to 20. Rats were sacrificed on the afternoon of P21 and uteri were removed and weighed. Values are group means ± s.e.m. ***p<0.005 compared to control. One factor ANOVA and *post hoc* Dunnett t-tests.

(10) The Effect of NMDA Treatment (P21 to P25) on Uterine Weight

From the results of *experiment 6* (Table 2) we know that a single daily injection of NMA (30 mg/kg) from P21 to P25 elicits the same degree of precocious sexual maturation as injections administered from P24 to P28 or P21 to VO. We examined the time course of the stimulatory effect of the early NMDA treatment on rising estrogen levels by measuring uterine weight on P26 and P29. A 2 factor ANOVA revealed significant effects of treatment ($F_{1,40}=5.353$; $p=0.0259$), age ($F_{1,40}=28.89$; $p<0.001$) and a treatment by age interaction ($F_{1,40}=5.385$; $p=0.0255$). At 26 days of age, the day after the last NMDA injection, the uterine weights of the NMDA treated and the control groups are not significantly different (NMDA: 53.5 ± 2.4 mg (10); control: 53.5 ± 5.9 (10)). However, at P29 *post hoc* Student's t-tests show a significant ($p<0.01$) increase in the uterine weight of NMDA-treated animals compared to controls (NMDA: 143.8 ± 13.5 mg (13); Control: 89.3 ± 16.0 (11)) (Fig. 14). Of the 13 rats in the NMDA group, 4 rats had reached VO and another 4 were in proestrus (large, fluid-filled uteri) at 29 days of age. In the control group (n=11), one rat had reached VO and another was in proestrus.
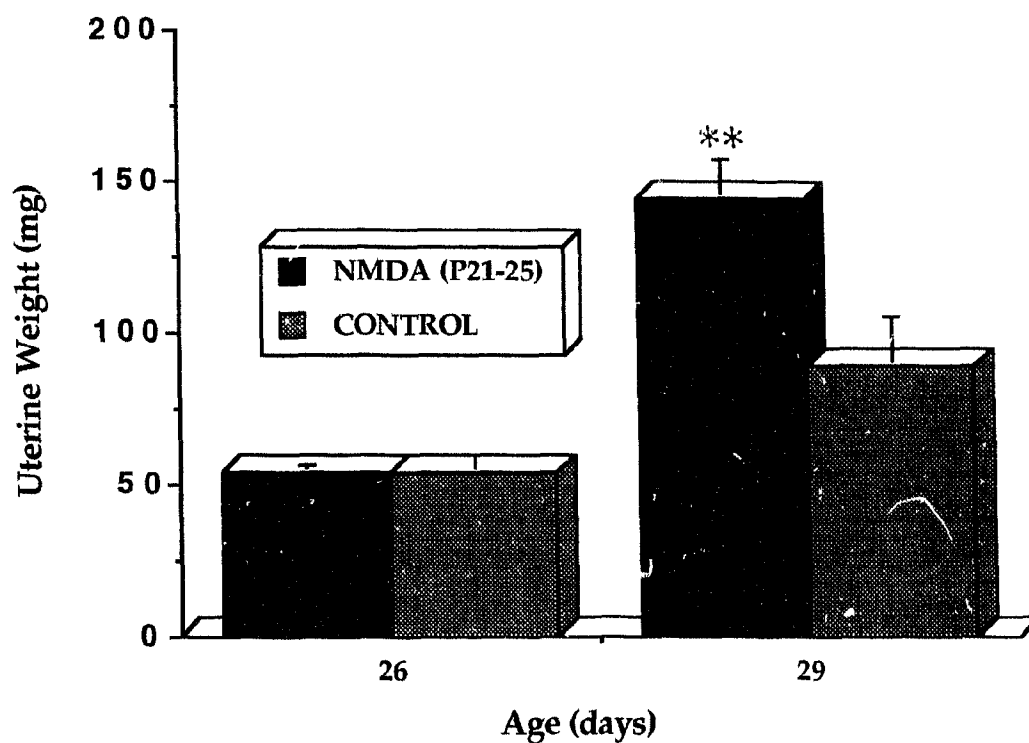
Figure 14. The effect of NMDA from P21 to 25 on uterine weight on P26 and P29.

Effect of daily NMDA (20 mg/kg; P21 to 25) or saline (0.1 ml; P21 to 25) injections (s.c.) on the uterine weight of P26 (n=10) and P29 (n=13) female rats. Rats were sacrificed on the afternoon of P26 or P29 and uteri were removed and weighed. Values are group means ± s.e.m. **p<0.01 compared to age-matched control. 2 factor ANOVA and *post hoc* Student t-tests.

(11) The Effect of Daily LHRH Injections on VO and Uterine Weight

Daily injections of LHRH (5 ng/100g; P24 to 28), at a dose which elicits an LH response in the same range as 20 mg/kg of NMDA, do not advance sexual maturation. The mean day of first ovulation in the LHRH-treated group is not significantly different from the control group but is significantly ($p<0.005$) different from the NMDA-treated group (NMDA: 30.0 ± 0.3 days (10); LHRH: 32.3 ± 0.3 (10); control: 32.0 ± 0.2 (10)) (Fig. 15). In contrast to NMDA treatment, daily LHRH injections do not significantly lower the body weight at first ovulation when compared to control rats (NMDA: 93.0 ± 0.9 g; LHRH: 110.6 ± 2.1 g; Control: 106.6 ± 1.8 g; $p<0.005$ compared to control). Similarly, the uterine weight of the LHRH-treated group is significantly ($p<0.005$) different from the NMDA-treated group but not the controls (NMDA: 128.2 ± 17.5 mg (11); LHRH: 69.9 ± 6.0 (11); control: 63.7 ± 5.7 (10)) (Fig. 16).
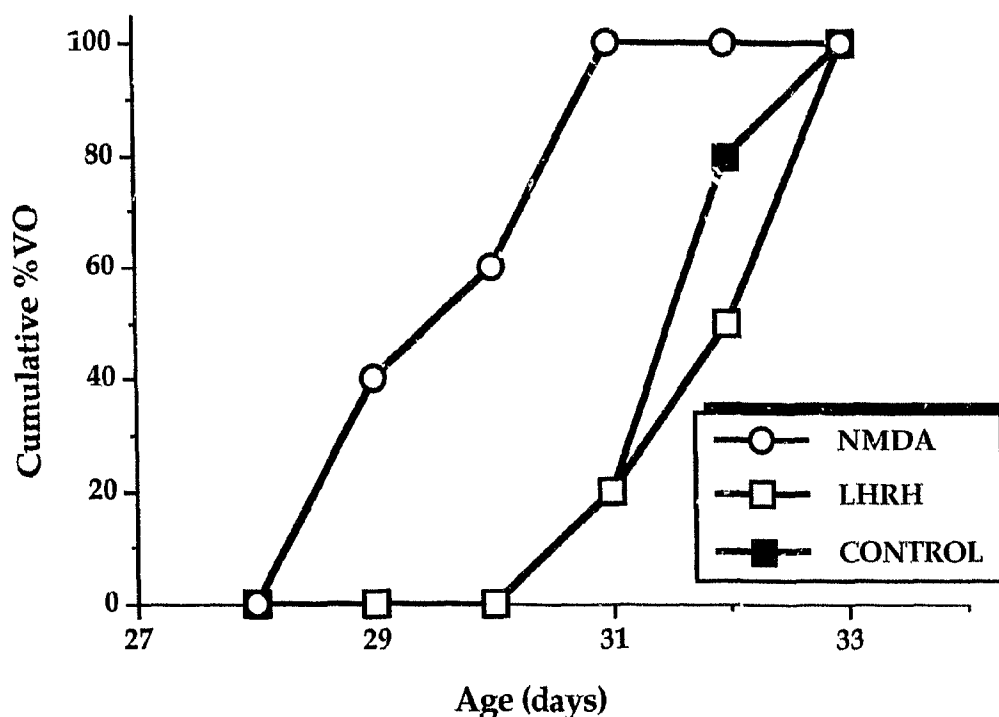
Figure 15. The effect of NMDA and LHRH treatment on the timing of VO.

A comparison of the effects of daily NMDA (30 mg/kg; n=10), LHRH (5 ng/100g; n=10) or 0.9% saline (0.1 ml; n=10) injections (s.c.) from P24 to P28 on the timing of VO in female rats. LHRH (5 ng/100g) gives an LH response in the same range as NMDA (20 mg/kg). Data are plotted as the cumulative percentage of rats showing VO at each age.
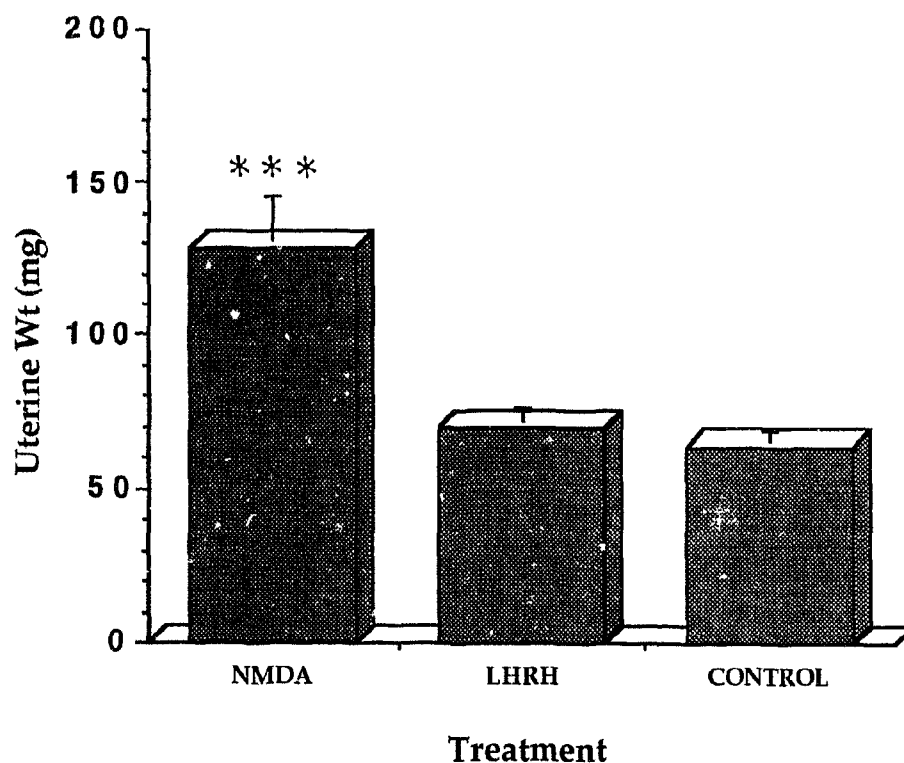
Figure 16. The effect of NMDA and LHRH on uterine weight.

The effects of daily NMDA (30 mg/kg; n=11), LHRH (5 ng/100g; n=11) or 0.9% saline (0.1 ml; n=10) injections (s.c.) from P24 to P28 on uterine weight (mg) on P29. LHRH (5 ng/100g) gives an LH response in the same range as NMDA (20 mg/kg). Values are group means ± s.e.m. ***p<0.005 compared to age-matched control. 1 factor ANOVA and *post hoc* Dunnett t-tests.

(12) The Effect of Bromocriptine on NMDA-Induced Early Puberty

Inhibition of prolactin secretion via the dopamine agonist, bromocriptine, slightly reduced the stimulatory effects of NMDA on sexual maturation in female rats. The mean day of first ovulation in the bromocriptine/NMDA-treated group is significantly ($P<0.005$) greater than the NMDA-treated group but it is also significantly lower ($p<0.001$) than controls (NMDA: 28.9 ± 0.3 days (10); bromocriptine/NMDA: 30.4 ± 0.2 (10); bromocriptine: 32.8 ± 0.4 (10); Control: 32.8 ± 0.4 (10)) (Fig. 17). However, *post hoc* Dunnett t-tests do not detect a significant difference in the mean body weight at VO of the Bromocriptine/NMDA group compared to the NMDA group (NMDA: 86.3 ± 2.1 g; bromocriptine/NMDA: 91.3 ± 1.3; Bromocriptine: 105.4 ± 2.0; Control: 110.2 ± 2.8). Interestingly, bromocriptine treatment alone from P24 to P28 had no effect on the mean day of VO or the mean body weight at VO compared to the control group.
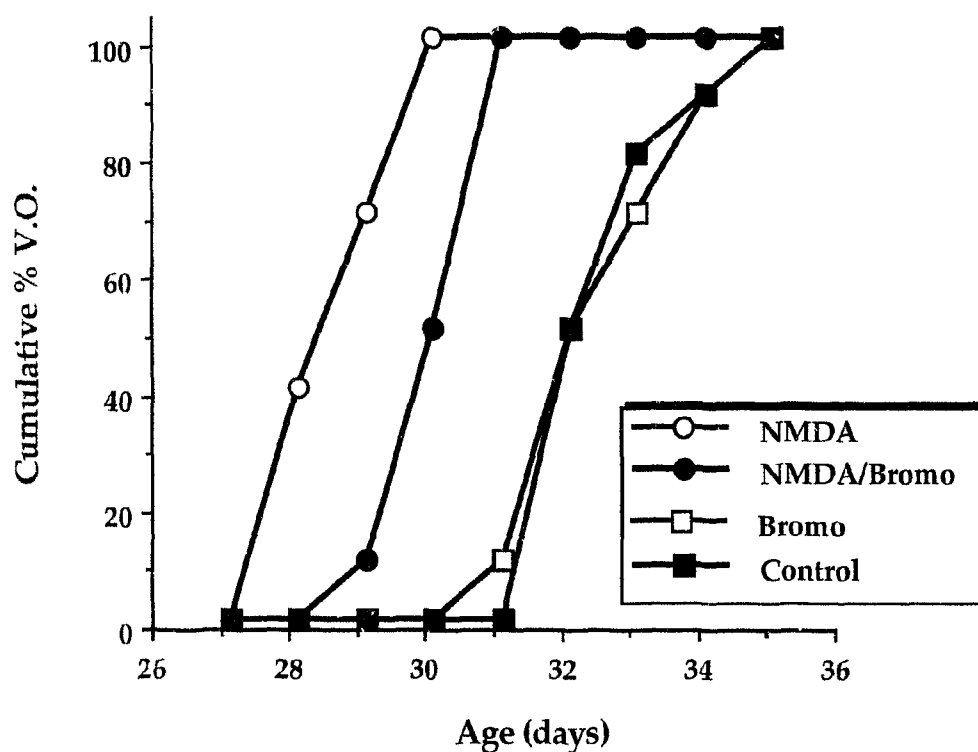
Figure 17. The effect of bromocriptine on NMDA-induced precocious puberty.

Rats were injected (s.c.) with a suspension of bromocriptine (0.5 mg) or 0.9% saline (0.1 ml) in the morning from P24 to 28. In the afternoon during this treatment period rats were injected (s.c.) with either NMDA (20 mg/kg) or 09% saline (0.1 ml). Data are plotted as the cumulative percentage of rats showing VO at each age (n=10/group).

(13) The Combined Effect of Neonatal MSG and Prepubertal NMDA on Sexual Maturation

Neonatal MSG, prepubertal NMDA and the combined (MSG/NMDA) treatment induce precocious puberty (MSG/NMDA: 29.1 ± 0.2 days (n=14); NMDA: 30.5 ± 0.4 days (n=14); MSG: 31.2 ± 0.4 days (n=14); Control: 34.6 ± 0.5 days (n=4)) (Fig. 18). *Post hoc* Dunnett t-tests reveal that the combination of neonatal MSG and prepubertal NMDA treatment causes a significantly greater precocity than neonatal MSG (MSG/NMDA vs. MSG; p<0.005) or prepubertal NMDA (MSG/NMDA vs. NMDA; p<0.025) alone. Neither MSG, NMDA nor the combined treatment affected growth rate but, compared with controls, treated rats reach VO at significantly (p<0.005) lower body weights (MSG/NMDA: 92.0±1.6 g; NMDA: 106.1±2.9 g; MSG: 102.7±1.8 g; Control: 122.8±2.6 g). Combined neonatal MSG and prepubertal NMDA treatment appears to have an additive effect, significantly (p<0.005) lowering the mean body weight at first ovulation compared to either treatment alone .
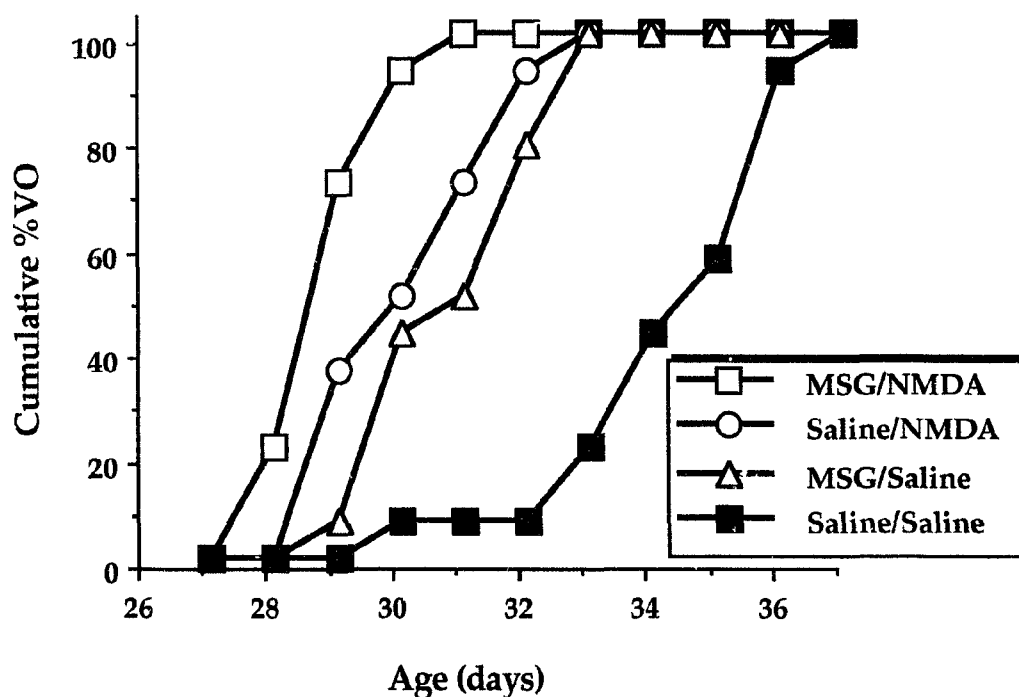
Figure 18. The combined effect of neonatal MSG and prepubertal NMDA on the timing of VO.

On P2, litters of female pups were treated with MSG (2 mg/g; 0.1 ml; s.c.) or 0.9% saline (0.1 ml; s.c.). and beginning on P24, the MSG-treated rats and saline-treated rats were injected (s.c.) daily (1100 h) with NMDA (20 mg/kg) or 0.9% saline (0.1 ml) until VO was observed. Data are plotted as the cumulative percenta of rats showing VO at each age (n=14/group).

II. LH Experiments

(1) The Effect of Different NMA Doses on LH secretion

The dose of NMA required to produce half maximal stimulation of LH release (ED_{50}) was approximately 6 mg/kg, about 1.5 times the value previously reported for NMDA. Maximal LH secretion is observed between 10 and 30 mg/kg of NMA. This is virtually the same as the dose of NMDA required to produce maximal LH secretion at this age (MacDonald and Wilkinson, 1990).

(2) Alterations in the LH Response to NMDA During NMDA-Induced Puberty

The purpose of this experiment was to investigate whether the LH response to NMDA changes during the series of daily injections used to initiate precocious VO. MacDonald and Wilkinson (1992) have shown that the LH response to NMDA (15 mg/kg) is maximal between P15 and P27 and it declines rapidly as the female rat approaches puberty (no LH response on day 32). Figure 19 demonstrates that this natural decline in the LH response to NMDA occurs sooner in the group receiving daily injections of NMDA from P24 than the control group. Most noticeable is the significant difference in NMDA-induced LH response on P28. The LH response to the third NMDA injection on P26 was greater than the LH response to the first injection of NMDA on the same day. However, this potentiation was evident in only 2 out of 4 experimental trials. A two factor ANOVA revealed significant effects of treatment ($F_{1,308} = 7.25$; $p < 0.01$) and age ($F_{4,308} = 18.69$; $p < 0.001$) but not a significant treatment by age interaction ($F_{4,308} = 2.06$).
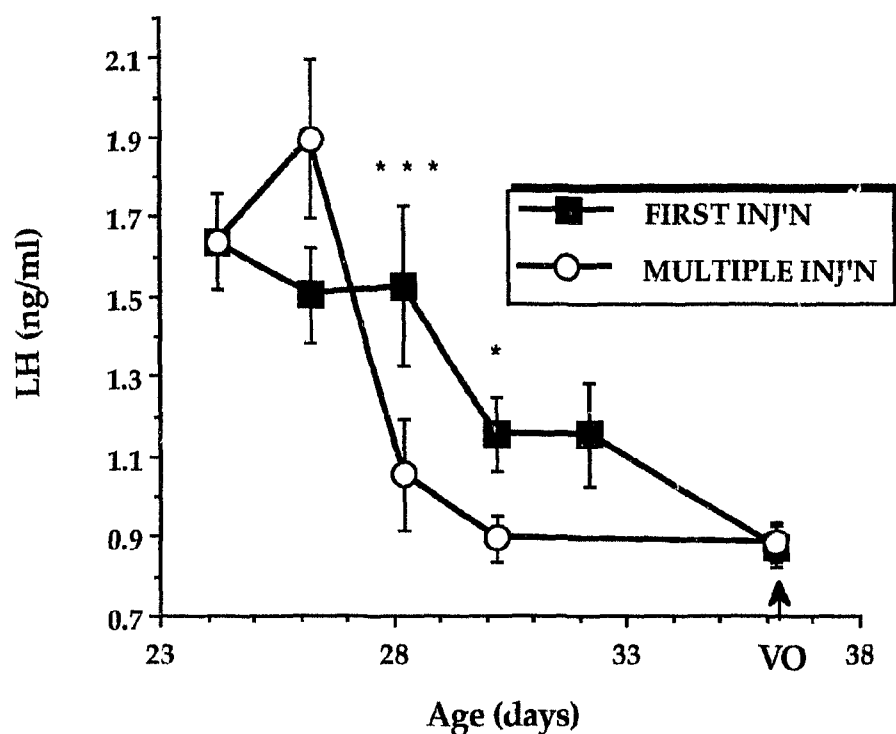
Figure 19. Effect of daily injections of NMDA on the ontogeny of the LH response to NMDA.

Rats were injected (s.c.) with either NMDA (20 mg/kg) or 0.9% saline (0.1 ml) from P24 to 28. At different ages from P24 to VO, subgroups of saline- and NMDA-treated rats were injected (s.c.) with NMDA (20 mg/kg). Rats were sacrificed 8 minutes after injection on P24 (first inj'n: n=35), P26 (first inj'n: n=33; third inj'n: n=26), P28 (first inj'n: n=27; fifth inj'n: n=27), P30 (first inj'n: n=37; seventh inj'n: n=32), P32 (first inj'n: n=10) and on the day of first ovulation (first inj'n: n=18; multiple inj'n: n=48). *p<0.05 and ***p<0.005 compared to the corresponding age-matched control group; 2 factor ANOVA (omitting day 32 data) and *post hoc* Student's t-tests. Values are mean ± SEM.

(3) LH Dose Response to NMDA on P28 in Control and NMDA-Treated Rats

The previous experiment showed that when comparing control and NMDA treated groups, the largest difference in LH response to NMDA occurs on day 28. This difference was further characterized by generating an LH dose response curve to NMDA in control and treated rats on day 28. Figure 20 shows that at 10 and 20 mg/kg of NMDA, rats which have received previous daily injections of NMDA (20 mg/kg; day 24-27) release significantly (p<0.01) less LH than the control group. A two factor ANOVA reveals significant effects of treatment ($F_{1,108}$=9.033; p<0.005) and dose ($F_{3,108}$=14.646; p<0.005) on LH response but not a significant treatment by dose interaction ($F_{3,108}$=1.694).

(4) LH Dose Response to LHRH on P28 in Control and NMDA-Treated Rats

The decline in NMDA-induced LH secretion following daily injections of NMDA may occur due to a decrease in pituitary (LHRH) or hypothalamic (NMDA) responsiveness. To test this hypothesis, the LH response to LHRH was evaluated in rats (P28) that had received four previous injections of NMDA (20 mg/kg; from day 24 to 27). As shown in Figure 21, the control and NMDA-treated groups had a similar LH response to increasing doses of LHRH. A two factor ANOVA did not reveal a significant treatment effect ($F_{1,83}$=2.099; p=.15) or a treatment by dose interaction ($F_{3,83}$=1.539; p=.21) on LH response. In addition, *post-hoc* Student's t-tests did not reveal a significant difference in LH response to LHRH at any dose. This suggests that NMDA treatment does not reduce pituitary responsiveness to LHRH.
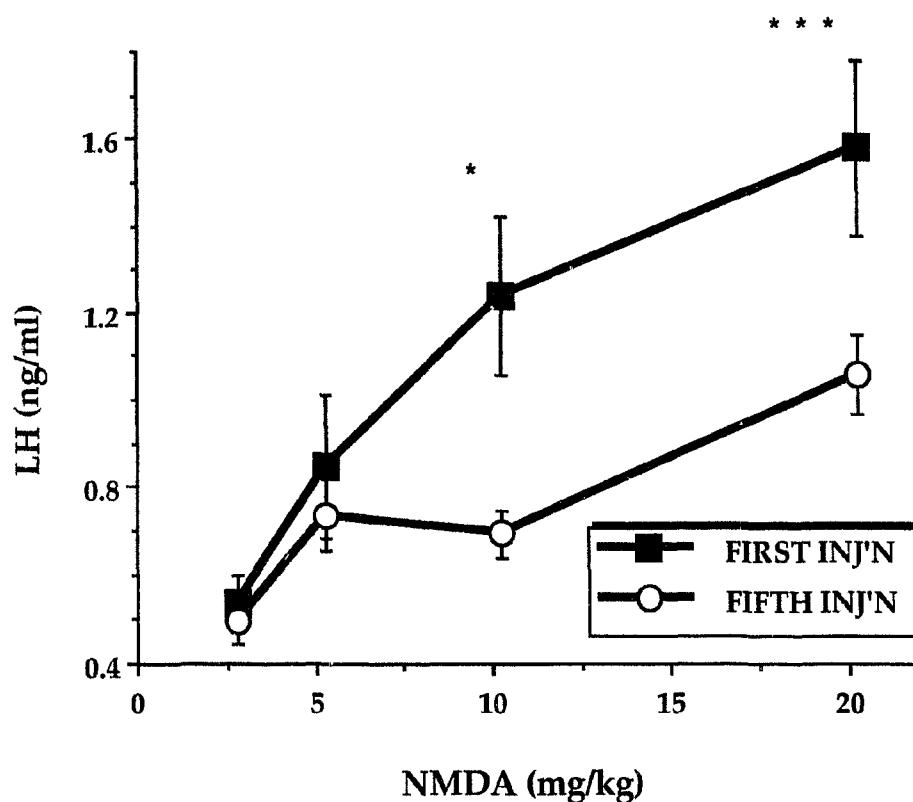
Figure 20. Characterization of the change in the LH response to NMDA on P28 in rats that have received four previous daily injections of NMDA.

LH was measured in immature female rats which had received daily injections (s.c.) of saline (0.1 ml; P24 to 27) or NMDA (20 mg/kg; P24 to 27) 15 min. following an injection of one of various doses of NMDA (2.5 mg/kg: first inj'n: n=10, fifth inj'n: n=10, 5 mg/kg: first inj'n: n=10, fifth inj'n: n=11, 10 mg/kg: first inj'n: n=10, fifth inj'n: n=11; 20 mg/kg: first inj'n: n= 27, fifth inj'n: n= 27). Values are mean ± s.e.m. *p<0.05; ***p<0.005 compared to age-matched control group; 2 factor ANOVA and *post hoc* Student's t-tests.
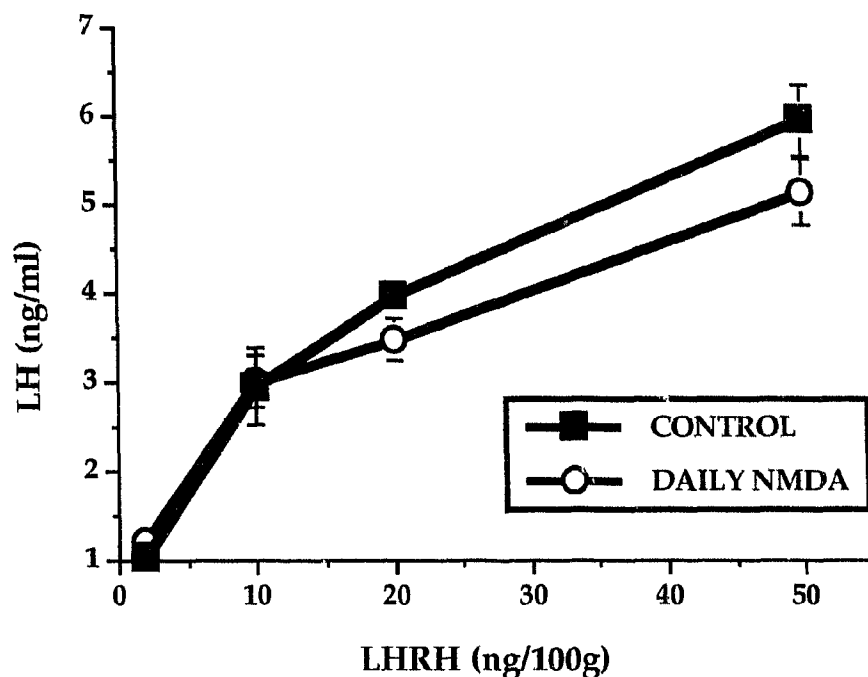
**Figure 21.** Effect of previous daily injections of NMDA on the LH (pituitary) response to various doses of LHRH on P28.

Immature female rats which had received daily (1100 h) injections (s.c.) of saline (0.1 ml) or NMDA (20 mg/kg) from P24 to 27 were injected (s.c.) with various doses of LHRH (2.5 ng/100g: control: n=18, daily NMDA: n=10; 10 ng/100g: control: n=8, daily NMDA: n=10; 20 ng/100g: control: n=15, daily NMDA: n=10; 50 ng/100g: control: n=10, daily NMDA: n=10) on P28. Rats were sacrificed 15 min. post-injection and blood was collected. Values are mean LH levels per group ± s.e.m. No significant difference between groups. 2 factor ANOVA and *post hoc* Student's t-tests.

(5) The Time Course of the LH Response to NMDA and LHRH after Previous NMDA and LHRH Treatment

The time course of the LH response to an injection of NMDA on P28 appears to be altered by previous NMDA treatment (20 mg/kg; P24 - P27). A two factor ANOVA of LH levels reveals significant main effects of treatment ($F_{1,88}=7.462$; $p=0.076$), time ($F_{4,88}=27.002$; $p<0.001$) and a treatment by time interaction ($F_{4,88}=2.653$; $p=0.0382$). Figure 22 shows that the maximal LH response to NMDA occurs 8 min. post-injection and that LH secretion returns to normal basal levels by 30 min. Interestingly, the LH response of the group receiving NMDA for the first time appears to rebound at the 60 min. time point and a *post hoc* Student's t-test shows that serum LH levels are significantly higher than the 30 min. time point ($p<0.005$) and the NMDA (x5) group at 60 min ($p<0.025$). Consistent with the results for *experiments LH2 and LH3*, there is a significant ($p<0.025$) decline in the LH response to NMDA at 8 min. post injection in the group receiving NMDA for the 5th time.

Four previous daily injections of LHRH significantly changes the LH response to LHRH (5 ng/100 g) on P28 (Figure 23). A two factor ANOVA detects significant effects of treatment ($F_{1,88}=29.49$; $p<0.001$), time ($F_{4,88}=59.223$; $p<0.001$) and a treatment by time interaction ($F_{4,88}=4.175$; $p=0.0038$). Both treatment groups reach peak LH levels 15 to 20 min. after injection and LH levels return to normal basal levels by 60 min. However, *post hoc* Student's t-tests reveal that there is significantly more serum LH in the LHRH (x5) group at 8 ($p<0.001$), 15 ($p<0.01$) and 30 ($p<0.025$) min. post-injection compared to those receiving LHRH for the first time.
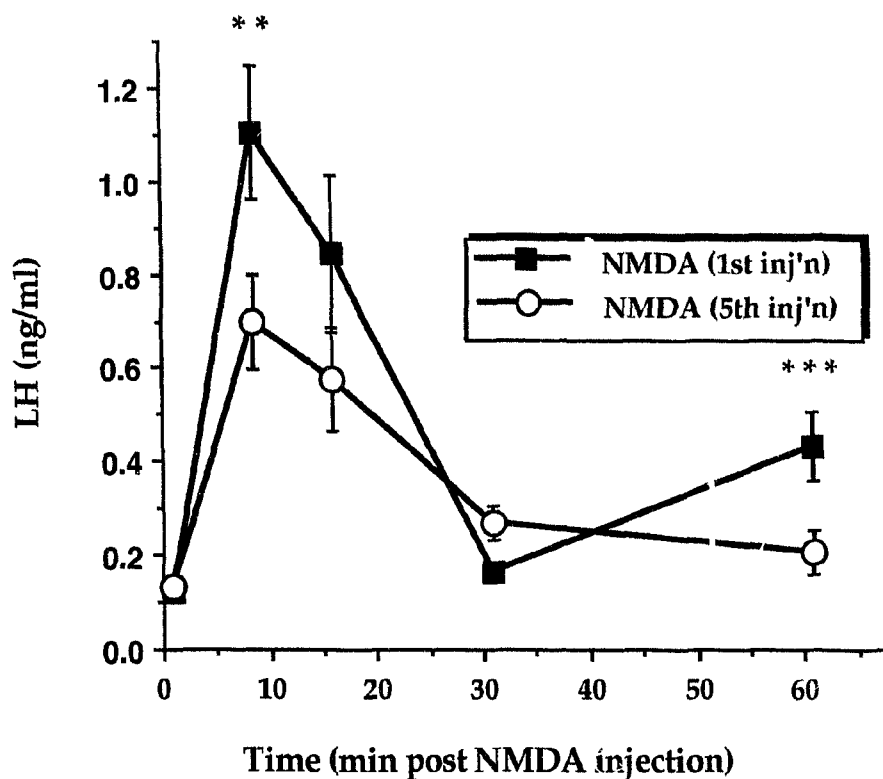
Figure 22. Effect of four previous daily injections of NMDA on the time course of the LH response to NMDA on P28.

Immature female rats were treated with NMDA (20 mg/kg; s.c.) or 0.9% saline from P24 to P27. On P28, both NMDA-treated and control rats were injected (s.c.) with NMDA (20 mg/kg) and were sacrificed at several time points after injection (0, 8, 15 , 30 , 60 min; n=9-10/time pt.). Values are mean LH levels per group ± s.e.m. **p<0.025; ***p<0.05 compared to control (first inj'n). 2 factor ANOVA and *post hoc* Student's t-tests.
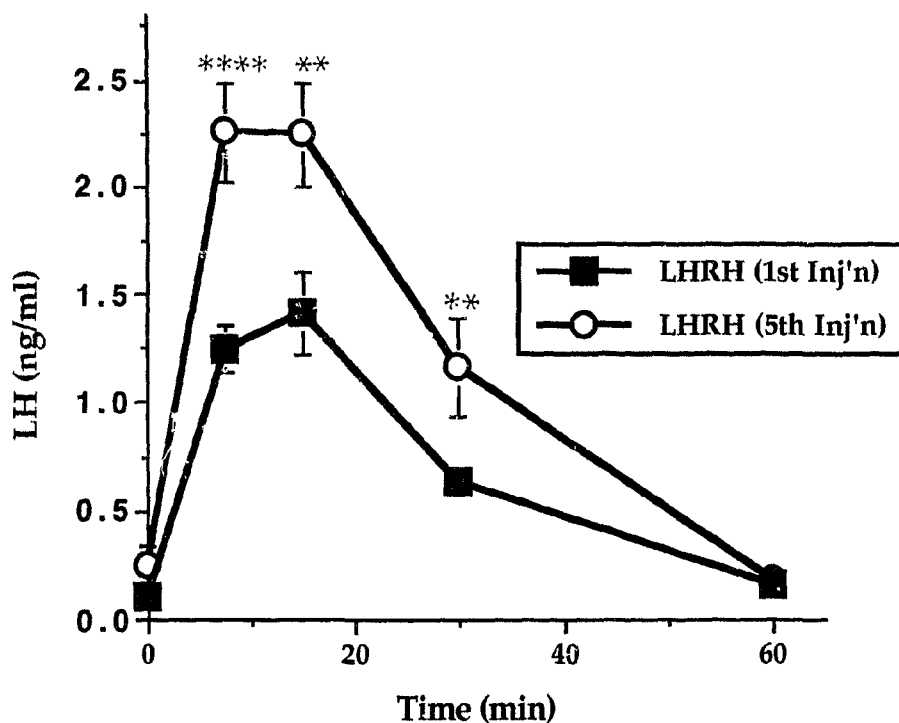
Figure 23. Effect of four previous daily injections of LHRH on the time course of the LH response to LHRH on P28.

Immature female rats were treated with LHRH (5 ng/100g; s.c.) or 0.9% saline from P24 to P27. On P28, both LHRH-treated and control rats were injected (s.c.) with LHRH (5 ng/100g) and were sacrificed at several time points after injection (0, 8, 15 , 30 , 60 min; n=9-10/time pt.). Values are mean LH levels per group ± s.e.m. **p<0.025; ****p<0.001 compared to control (first inj'n). Two factor ANOVA and *post hoc* Student's t-tests.

Discussion

Van den Pol and Trombley (1993) have suggested that glutamate is the major excitatory neurotransmitter in the neuroendocrine hypothalamus. They go on to state that "it is probably the major excitatory neurotransmitter inducing release of neuroendocrine hormones from axons terminating in both the median eminence and the posterior pituitary" (Van den Pol et al., 1990). Therefore, it is not surprising that glutamate acting through its NMDA receptor has been established as a critical component of the neuronal mechanism controlling LH secretion and the onset of puberty in the rat.

Acceleration of sexual maturation in the female rat by the EAA NMDA was first reported by Urbanski and Ojeda (1987). Despite the significance of these findings, the procedure used in their study was complicated (20 mg/kg NMDA; two - 1 min. pulses/hr. between 1300 and 1700 hrs. from P26 to 29) and costly (microprocessor-driven pulsatile intravenous administration; see equipment described in Urbanski et al., 1984) making it an impractical model for general research use. Using a much simpler and inexpensive method, our laboratory has reported that single daily s.c. injections of NMDA (15 mg/kg/day from P27 to VO) are effective in synchronizing first ovulation and can slightly (1-2 days) accelerate sexual maturation (MacDonald and Wilkinson, 1990). The single daily injection schedule was subsequently used by Urbanski (1990), to increase serum LH and testosterone levels in short-day, non-breeding male Syrian hamsters, and by Nyberg et al. (1993) and Brann et al. (1993c) in the rat. The experimental results presented here demonstrate that a higher dose of NMDA (20 mg/kg) or NMA (20 and 30 mg/kg), initiated earlier in development, can significantly advance puberty (3 to 4 days).

The puberty-inducing effect of a single daily injection of NMDA is independent of the time of day it is applied. In other words, the stimulatory

effect of NMDA on sexual maturation is not limited to late in the light phase when prepubertal LH pulses and minisurges normally begin to appear (Urbanski and Ojeda, 1985). This result was surprising to us because MacDonald (1989; Ph.D. dissertation) found that daily administration of the noncompetitive NMDA antagonist, MK-801 (0.1 mg/kg; s.c.; from P27 to VO), significantly delayed puberty but its actions were regulated by the clock. MK-801 injections at 0900 hrs. had no effect on the timing of first ovulation but as injections were initiated later in the day, puberty was delayed and the most pronounced effects were observed when injections were administered close to the afternoon increase in pulsatile LH secretion. This demonstrates that endogenous glutamate acting through NMDA receptors is involved in the onset of puberty perhaps by regulation of the neuronal mechanism generating afternoon pulses of LH. MK-801 is use-dependent (i.e. it can only block the NMDA receptor when stimulated with endogenous glutamate) but NMDA can stimulate LHRH release presumably at any time. Also, MK-801 is lipid soluble and affects whole brain whereas it is unlikely that NMDA crosses the blood brain barrier (BBB).

Although NMDA treatment from P27 to VO or P29 to VO has only a minimal effect on the advancement of sexual maturation, it does have a pronounced effect on the synchronization of the day of first ovulation. On the other hand, NMDA injections initiated earlier in development (before 24 days of age) have a significant effect on precocity but very little, if any, effect on synchronicity. We have no explanation for these ambiguous findings, particularly since the LH response to NMDA is attenuated during the treatment period close to puberty (see below).

NMDA-stimulated early puberty appears to be dose-related. A small dose of NMDA (5 mg/kg) significantly decreases the average body weight at VO of treated animals but although the mean day of VO is earlier, it is not significantly

different than controls. In our model of puberty, the maximal advancement of first ovulation (3 to 4 days) occurs using a daily dose of 20 mg/kg. Doubling or tripling the dose had no further effect on the timing of the mean day of VO. We compared the stimulatory effects of one vs. three separate injections of NMDA (20 mg/kg/injection) but have yet to try a single higher (60 mg/kg) dose of NMDA. We hesitated in doing this due to the neurotoxicity reported at this dose (Price et al., 1978a) and the adverse behavioural effects of the drug (MacDonald and Wilkinson, 1992).

Our experiments with the D,L form of the agonist (NMA) demonstrated that NMA was as effective as the more costly NMDA at inducing precocious VO. This enabled us to determine optimal conditions of drug treatment. Thus, it is clear from our data that continued daily injections until the time of VO (ie. P24 to VO) is not necessary to induce maturation since injections from P24 to P28 or even P21 to P25 are equally effective. NMDA treatment of younger rats (P16 to P20) had no effect on the mean day of VO. However, experiment 9 showed that the ovaries of P16 rats can respond to enhanced gonadotrophic drive (PMSG) with increased estrogen production which indicates that the immaturity of the rat ovary does not limit NMDA-induced early puberty.

These data suggest that there is a critical period where glutamate receptor stimulation becomes capable of inducing precocious puberty (approximately P21 to P29). It is unknown whether this critical period is related to NMDA receptor affinity or density in the developing hypothalamus. NMDA-sensitive glutamate binding is very low in hypothalamus when examined autoradiographically (Smyth, Jacobson, Wilkinson; unpublished data). In contrast to this, Meeker et al. (1994) used saturation binding analysis to quantify the glutamate receptor in the hypothalamus and reported relatively high densities of NMDA and non-NMDA receptors. Overall, there are more NMDA than non-NMDA receptors and this is

especially true in the ventromedial hypothalamic nucleus (VMN), dorsomedial nucleus (DMN) and ARCN. High levels of an NMDA receptor subunit (NR1) mRNA have also been identified in rat and hamster MBH (Monyer et al., 1992; Watanabe et al., 1993; Urbanski et al., 1994). More specifically, high levels of NR1 mRNA have been localized to the VMN of the rat and to the VMN-ARCN area of the hamster (Watanabe et al., 1993; Urbanski et al., 1994). NMDA.R1 appears to be developmentally regulated in areas of the brain such as cerebral cortex, hippocampus and cerebellum (Pujic et al., 1993; Monyer et al., 1994) and there is also some evidence that NMDA.R1 expression in the hypothalamus is similarly regulated. For instance Della Vedova et al. (1994) have shown relatively steady state levels of two NMDA.R1 splice variants in the hypothalamus through development with the exception of a significant increase in splice variant, NMDA.R1$_{1xx}$ at P15 and P18.

It would be of interest to characterize the ontogenic changes in NMDA receptor expression and sensitivity in female rats. Relatively simple techniques such as immunocytochemistry (ICC; polyclonal NR1 antibody from Pharmingen, San Diego, CA), in situ hybridization histochemistry (ISHH; oligonucleotide cDNA as described in Watanabe et al., 1993) and NMDA receptor binding assays (ligand [^{3}H]-CGP 39653) could be used to examine the developmental changes in the expression, density and sensitivity of the NMDA receptor. Several research groups have attempted to examine this issue already. Brann et al. (1993b) have reported that neither NMDA nor kainate receptor binding in membrane preparations from the whole hypothalamus (rather than discrete nuclei) changes during sexual maturation of the female rat. However, this group examined glutamate receptor binding on P20 and P32-35 and may have missed significant changes in the density or sensitivity of the NMDA receptor between P21 and P31

- the approximate time identified in this thesis as the critical period of NMDA receptor-mediated induction of early puberty.

Ontogenic changes in the activity of NMDA may be explained not only by changes in the density and sensitivity of the receptor but also by changes in amino acid levels in the hypothalamus of female rats (Goroll et al., 1994; Otero Losada et al., 1993). This proposal is not without precedent as Frankfurt et al. (1984) reported changes in GABA and glutamate concentrations in several areas of the CNS through the estrous cycle of the adult rat. Otero Losada et al. (1993) used HPLC to quantifiy amino acid levels in the MBH/POA of juvenile female rats. They found that endogenous levels of aspartate (ASP), glutamate (Glu), and glycine (Gly) were higher at P30 than P16 but the taurine (Tau) concentration decreased between P16 and P30. Treatment with NMDA stimulated an increase in Glu at P16 and an increase in Glu and Asp at P30; both of which were inhibited by MK-801. Interestingly, MK-801 alone had no effect at P16 but significantly decreased the concentrations of Glu, Asp, Tau and Gly levels at P30. The results reported by Goroll et al. (1994) also show that specific amino acid levels increase with age (Glu, Gly, Asp) and Tau concentration decreases with age. However, they reported that the most pronounced changes occur in the POA not in the MBH. Otero Losada et al. (1993) hypothesize that "the positive interaction between NMDA receptors and amino acid levels may be involved in the neuroendocrine events that result from the increase in EAAergic activity related to the onset of puberty".

Another possible mechanism that might account for a critical period in NMDA-induced puberty is suggested by the work of Bourguignon et al. (1990a,b, 1992a,b). In immature male rats there appears to be an effective NMDA receptor mediated inhibition of LHRH secretion at P15 since MK-801 (0.001 mg/kg) was able to induce early pubertal development in male rats (Bourguignon et al.,

1992b). By P25 a stimulatory effect of NMDA on LHRH secretion appears. It is not known whether a similar inhibitory mechanism exists in the female rat, although even at P15 there is a robust LH response to NMDA (MacDonald and Wilkinson, 1992). Cicero et al. (1988) have also shown that in male rats the LH response to NMDA at P15 is stimulatory. It is possible that NMDA treatment from P16 to 20 occurs at a neutral junction between the inhibitory and stimulatory effects of NMDA on LH secretion and sexual maturation.

The critical period of NMDA receptor stimulation of precocious puberty seems to begin immediately after weaning. Is this a coincidence or could suckling or perhaps a substance in milk counteract the effects of NMDA in these preweanling pups? We know that lactation, mediated by prolactin, uncouples the activation of the NMDA receptor from LH secretion (Abbud et al., 1992, 1993) in the mother. Does suckling also have a physiological effect on the pups which could interfere with NMDA-stimulated sexual maturation?

Previous work in our laboratory has demonstrated that the LH response to NMDA declined with the approach of spontaneous first ovulation (MacDonald and Wilkinson, 1990). Our present results indicate that NMDA-induced precocious puberty accelerates this reduction in LH responsiveness to NMDA and this is most apparent on P28. Repetitive daily treatment with NMDA does not affect the time-course of the LH response. Since the pituitary responds normally to LHRH in NMDA-treated rats on this day, the attenuated LH dose-response to NMDA suggests that the hypothalamus is somehow desensitized to the glutamate agonist. The only similar report to ours was published by Schainker and Cicero (1980) stating that daily NMA treatment of male rats enhanced the subsequent LH response to the drug. In two of the four trials of this experiment we saw a significant increase in the LH response to NMDA on P26 but this was not consistent. Interestingly, at VO, both NMDA- and saline-

treated groups release the same amount of LH in response to NMDA suggesting that the mechanism of LH secretion is functionally the same in treated and control animals at this point of development.

The attenuation of NMDA-induced LHRH release may be mediated by the hormone LHRH in an autocrine feedback mechanism (Bourguignon et al., 1994). Breakdown products of LHRH inhibit NMDA-evoked LHRH secretion from retrochiasmatic hypothalamic explants of young adult male rats. In addition, Bourguignon et al. (1994) have demonstrated that the LHRH breakdown product 1-5LHRH causes a dose-related reduction in the binding of [^{3}H]-glutamate to hypothalamic membrane preparations.

Even though multiple injections of LHRH do not mimic the effects of NMDA on VO, there is a small increase in responsiveness of the pituitary to LHRH. NMDA treatment does not alter the responsiveness of the pituitary to LHRH but there is some evidence indicating that NMDA may act directly on the pituitary. Two recent articles reported glutamate receptor binding and NMDA.R1-li in the anterior and posterior pituitary (Meeker et al., 1994; Petralia et al., 1994). This is an intriguing observation as this area was previously shown to be unresponsive to glutamate stimulation (Schainker and Cicero, 1980). NMDA-induced LH secretion is believed to be mediated by hypothalamic LHRH release because *in vitro* exposure of rat and primate pituitaries to NMA does not stimulate LH release (Tal et al., 1983) and NMDA-induced LH release is prevented by pretreatment with an LHRH antagonist (Cicero et al., 1988; Urbanski, 1990). NMDA.R1-li occurs in scattered coarse-grained cells of the anterior pituitary. There is some indication that these NMDA receptor-positive pituitary cells may be somatotrophs. Lindström and Ohlsson (1992) reported that NMDA treatment of cultured male rat somatotrophs release growth

hormone (GH) in a dose-dependent manner which was blocked by NMDA receptor antagonists AP7, MK-801 and dextromethorphan.

Oster and Schramm (1993) have recently shown that NMDA treatment of cerebellar granule cells in culture induces a strong down-regulation of the NMDA receptor. They postulate that "the *in vivo* level of NMDA receptor activity is down-regulated to the desired level, following glutamatergic synaptogenesis" (Oster and Schramm, 1993). The effect of glutamate on neuronal plasticity has been well researched (see Introduction) I have previously hypothesized that NMDA may stimulate the maturation of the hypothalamic wiring pattern in our model of precocious puberty. Extrapolated from Oster and Schramm (1993), the attenuation of the LH response to NMDA seen in our animals as they approach first ovulation may be related to post-synaptogenic down-regulation of the NMDA receptor.

It is possible that the decline in NMDA-induced LH secretion is a necessary event in sexual maturation. Why NMDA should become less able to release LHRH before puberty is unclear but parallels a diminished ability of MK-801 to inhibit LHRH secretion *in vitro* (Bourguignon et al., 1992b). This modification was observed in castrate rats, suggesting that gonadal steroids may not be responsible. Finally, the attenuation of the NMDA activation of LHRH release provides an explanation for why MK-801, an antagonist of the NMDA receptor, can delay puberty but not completely block it (MacDonald and Wilkinson, 1990). This inability of MK-801 to prevent sexual maturation probably reflects a reduction in NMDA receptor stimulation and the appearance of another, non-NMDA, excitatory drive.

The kainic acid (KA) receptor has also been implicated in the control of LH secretion. Price et al. (1978a) reported a dose dependent rise in KA-induced LH release in juvenile male rats. In addition, Abbud and Smith (1991) have

reported that pulses of kainate administered to cycling adult rats stimulates LH release but the response is decremental with repetitive injections of the drug. In preliminary work we have shown that a prepubertal decline in LH response to a specific kainate agonist (domoic acid; DA) also occurs in female rats (MacDonald, Smyth and Wilkinson; unpublished observations). Blockade of non-NMDA receptors with DNQX inhibits the steroid- and PMSG-induced LH surge (Brann et al., 1993b). However, the same group has shown that KA treatment does not accelerate sexual maturation nor does DNQX delay puberty (Brann et al., 1993c). Several *in vitro* studies in pubertal and adult male rats show that LHRH release can be regulated by kainate (Bourguignon et al., 1989a; Lopez et al., 1992). Recently, KA was reported to stimulate LH and FSH secretion from perifused anterior pituitary *in vitro* (Zanisi et al., 1994).

MSG treatment (2 mg/g on P2) of neonates is another reliable animal model of precocious puberty used by our laboratory (see MacDonald and Wilkinson, 1990). The results from experiment 13 illustrate that the combination of neonatal MSG and peripubertal NMDA treatment advances puberty significantly more than either treatment alone. The stimulatory effect of MSG on sexual maturation was unexpected as we anticipated that MSG treatment would remove an excitatory influence on sexual maturation (glutamate receptor positive neurons). MSG treatment may cause NMDA receptor "supersensitivity", amplifying the excitatory effects of the neurotransmitter. Alternatively, MSG may remove an inhibitory influence on sexual maturation. For instance we have shown that MSG treatment alters the concentration and binding of GABA in the MBH of peripubertal female rats (Jacobson, Smyth, Szerb and Wilkinson; unpublished data). In addition, MSG may reorganize the hypothalamic wiring pattern through the stimulation of synaptogenesis (see Introduction).

How does NMDA/NMA treatment advance sexual maturation? Clearly, NMDA induces a dose-related increase in LH levels and daily treatment results in rising serum estrogen levels (increased uterine weight) when administered from P24. Treatment with estradiol benzoate (EB) is a well known model used for the induction of precocious first ovulation (Ramirez and Sawyer, 1965) and may possibly account for the puberty-accelerating effects of NMDA. In contrast, when NMA is administered between P21 and P25, uterine weight is not significantly increased compared to controls immediately after discontinuation of injections (P26). However, mean uterine weight in the NMA-treated group is significantly elevated on P29 indicating that NMDA treatment has some latent effect on the hypothalamus or pituitary which leads to enhanced production of estrogens and increased uterine weight.

The stimulatory effect of NMDA on sexual maturation cannot be mimicked by a dose of LHRH which releases a similar amount of LH as NMDA. This suggests that NMDA performs another function in addition to that of releasing LHRH/LH. Little is known concerning NMDA-induced release of other pituitary hormones, particularly in immature rats. NMDA releases FSH in female monkey (Wilson and Knobil, 1982) and in male hamster (Urbanski, 1990) whereas in the rat data are conflicting. Brann and Mahesh (1991a,b) showed that an NMDA antagonist (MK-801) can block a progesterone-induced LH/FSH surge. In contrast, Lopez et al (1990), using a different antagonist (AP7), found no effect on FSH. NMDA increases plasma ACTH levels in male (Farah et al., 1991; Jezova et al., 1991) and in neonatal female rats (Bardgett et al., 1992). NMDA receptors may also regulate GH secretion in rats (Cocilovo et al., 1992), monkey (Medhamurthy et al., 1992) and in sheep (Estienne et al., 1989). For prolactin (PRL), the situation is also unclear. Arslan et al. (1991) report that NMDA can inhibit and stimulate PRL release whereas Jezova et al (1991) failed to

see an increase. Lactation and suckling also modify the effect of NMDA on PRL (Pohl et al., 1989). In the ram Lincoln and Wu (1991) report that NMDA stimulates PRL release in long days. PRL secretion is also seen in female monkeys in response to NMDA (Wilson and Knobil, 1982). Login (1990) has shown that GLU can stimulate PRL release directly from pituitary cells. Thus GLU receptors, possibly of the NMDA subtype, are intimately involved in the secretion of several pituitary hormones, one of which - PRL - is known to stimulate precocious puberty (Advis and Ojeda, 1978). Indeed, in our NMDA model of puberty, blockade of PRL with bromocriptine treatment reduces but does not prevent the stimulatory effects of the EAA. Therefore, NMDA-stimulated prolactin release may be partially responsible for the precocity.

A further mechanism by which NMDA could accelerate sexual maturation is by a direct trophic effect on the brain. Excitatory amino acids (EAAs) and especially GLU, can regulate differentiation and synaptogenesis in the developing nervous system (see Introduction). For example, *in vitro* studies show a trophic effect of low concentrations of GLU on neurite outgrowth and synapse formation (Aruffo et al., 1987; Mattson et al., 1988). *In vivo* NMDA treatment also increases synapse formation in occipital cortex of postnatal rats (Brooks et al., 1991) (for reviews see McDonald and Johnston, 1990; Constantine-Paton, 1990). If we also take into account evidence that NMDA can regulate nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) levels *in vitro* and *in vivo* (see eg. Zafra et al., , 1990, 1991; Amano et al., 1992) it is then possible to suggest that NMDA (and possibly other GLU agonists) could induce puberty via growth factors and synaptogenesis.

In summary, we have identified a critical period, approximately P21 to P29, during which precocious VO can be induced by stimulation of NMDA-type glutamate receptors. Subsequent to this period the LH response to NMDA is

severely attenuated in treated and control rats. The evidence suggests that the hypothalamic-pituitary-LH system becomes <u>less</u> sensitive to NMDA with the approach of puberty even though NMDA can induce precocious ovulation.

CHAPTER 3 - REGULATION OF *C-FOS* GENE EXPRESSION BY GLUTAMATE RECEPTORS

Introduction

I. Immediate Early Genes and their Actions

The immediate response of a neuron to stimulation consists of rapid, transcription-independent events. Longer-term responses consist of sustained, transcription-dependent phenotypic changes of the sort believed accountable for neural plasticity and learning. The mechanisms involved in synaptic transmission and membrane depolarization are fairly well understood but the molecular mechanisms involved in stimulus-transcription coupling which lead to long-term or permanent changes in neurons remain unclear.

At the cellular level, extrinsic stimulation influences the levels or activity of second messenger molecules such as cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP$_3$) and Ca^{2+}. Changes in second messenger molecules initiate short-term responses directly, and elicit long-term responses indirectly by way of adaptive regulator molecules (see Fig. 24). The molecules considered most likely to serve as adaptive regulators are the proto-oncogenes, such as *c-fos*, which encode proteins that modulate the expression of the genes involved in long-term cellular changes such as growth and proliferation (Curran and Morgan 1987).

Proto-oncogenes belong to a family of genes variously known as immediate early genes (IEGs) or early-onset genes that are activated rapidly (minutes) and transiently following stimulation in a variety of tissue types, including the CNS. IEGs encode proteins that control the expression of late response genes involved in the normal cellular processes of proliferation and differentiation (Sheng and Greenberg, 1990).

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CYTOPLASM   **NUCLEUS**

1   e.g. Steroids, retinoids thyroid hormones   Target genes

2   2ⁿᵈ messenger   Primary target genes

Immediate-early genes

3   Synthesis of transcription factor proteins   Secondary target genes
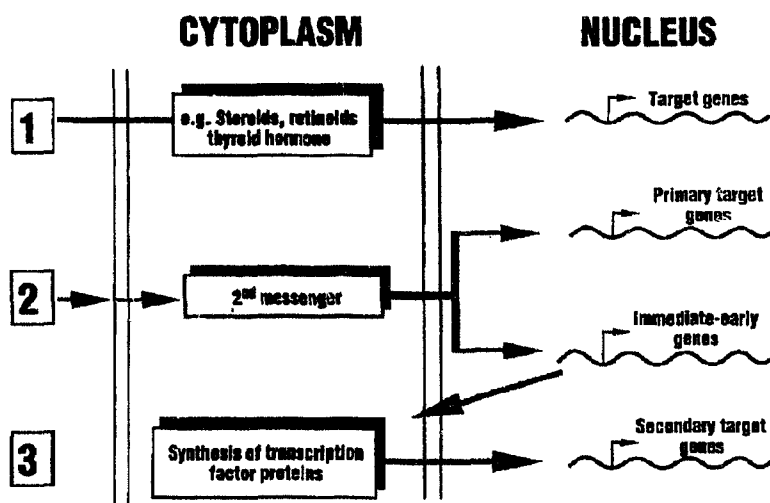
Figure 24. Regulation of gene expression by exogenous stimuli.

Extracellular stimuli can modulate gene expression in three basic ways. (1) Membrane-permeant molecules such as steroids, retinoids and thyroid hormones bind to receptors in the nucleus and directly alter target gene transcription. (2) Water-soluble molecules alter gene expression via activation of second messengers. Second messengers alter target gene expression either directly, by modifying the activity of constitutive transcription factors, or indirectly (3) by rapidly inducing cellular IEGs encoding transcription factors. (redrawn from Morgan and Curran 1989).

The chain of events resulting in cell growth begins with the binding of a growth factor to its receptor. This may occur either at the cell surface or inside the cell resulting in the mobilization of intracellular transducers (second messengers). Second messengers alter transcription activity within the cell by modifying the expression of active genes or by allowing new genes to be expressed. This process may involve activation of a nuclear transcription factor (third messenger) such as Fos, the protein product of the IEG c-fos (see Fig. 25).

II. The c-fos Proto-oncogene

c-fos is a member of the IEG family which also includes c-myc and c-jun (Sheng and Greenberg, 1990). IEGs share a number of common characteristics. In most cell types, basal levels of IEG expression are low or undetectable but stimulation by a variety of extracellular signals evokes rapid, transient transcription and induction of their mRNA and protein products (Sheng and Greenberg, 1990; Krukoff, 1994). Transcription activity does not require synthesis of new protein (Morgan and Curran, 1991), but protein synthesis is required to terminate transcription (Sheng and Greenberg, 1990).

In most situations, the time course of IEG activation is the same. Increases in c-fos mRNA occur within a few minutes of the onset of some stimuli (Greenberg and Ziff, 1984), reach maximal values at 30-45 min. and then decline rapidly with a half-life of approximately 12 min. (Morgan and Curran, 1991). The protein product of c-fos, Fos, has been detected from 20 min. to 24 hrs. following the onset of stimulation (Morgan et al., 1987; Le Gal La Salle, 1988; Menetry et al., 1989; Mugnaini et al., 1989; Elmquist et al., 1993). Following transcription, Fos is translocated to the nucleus where it forms a heterodimeric protein complex with Jun, the protein product of the IEG c-jun (Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Rauscher et al., 1988) (see Fig. 25). A sequence of amino acids called the "leucine zipper" is present in

both Fos and Jun and plays a central role in the formation of Fos/Jun heterodimers. The leucine zipper consists of an α-helical structure with leucine side chains aligned at regular intervals along one face of the helix (Morgan and Curran, 1991). Leucine side chains extending from the Fos and Jun helix are thought to interact with each other resulting in the formation of a Fos/Jun heterodimer somewhat resembling a coiled coil (Landschulz et al., 1988; Sheng and Greenberg, 1990). A sequence of amino acids located adjacent to the leucine zipper of Fos and Jun binds with high affinity and specificity to the nucleotide sequence -TGACTCA- (the so-called activator protein-1 (AP-1) binding site) and stimulates transcription of nearby genes (Franza et al., 1988; Sonnenberg et al., 1989a). The nature of the cellular response to *c-fos* activation is dependent upon the target genes within the host cell which are selected for regulation.

The protein products of all members of the families of IEGs to which *c-fos* and *c-jun* belong possess the leucine zipper dimerization motif (Sheng and Greenberg, 1990). These include *fos-B, fra-1,* and *jun-B.* Members of the Jun family of IEGs are able to dimerize with themselves or with any member of the Fos and Jun families (Sheng and Greenberg, 1990). Fos and Fos-related proteins, on the other hand, only form stable heterodimers with members of the Jun family (Cohen et al., 1989; Nakabeppu et al., 1988). Fos/Jun complexes activate transcription activity, but some heterodimeric combinations, such as Fos/Jun-B, are reported to repress gene transcription in some instances (Chiu et al., 1989; Schütte et al., 1989).
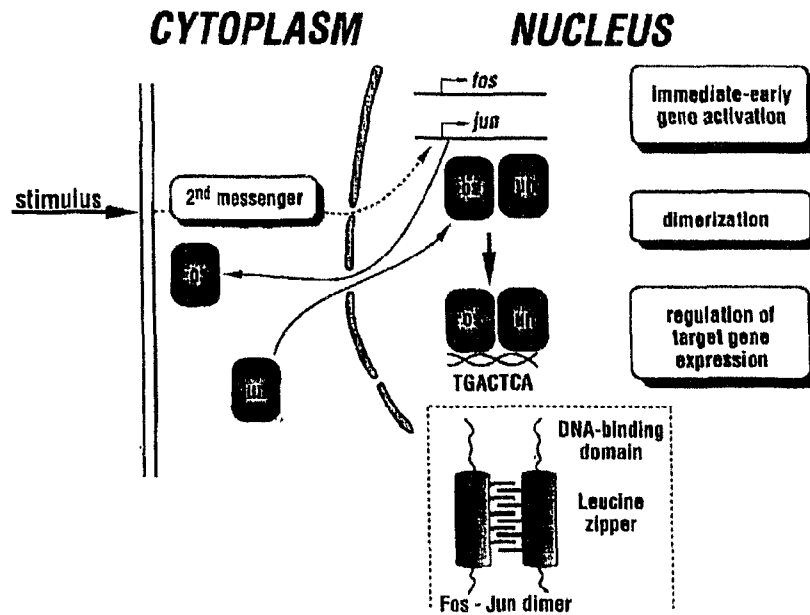
Figure 25. Role of Fos and Jun in nuclear signal transduction.

Extracellular stimuli bring about changes in second messenger levels which lead to an induction of both *c-fos* and *c-jun*. This activation involves distinct regulatory elements in the 5' flanking region of the two genes. Following translation in the cytoplasm, Fos and Jun (the proteins encoded by *c-fos* and *c-jun* respectively) are rapidly translocated to the nucleus where they form a heterodimeric complex. This complex binds with high affinity to the AP-1 DNA consensus recognition sequence (TGACTCA). The AP-1 regulatory element is known to exist in control regions of several genes and is essential for both basal and stimulated transcription. (redrawn from Morgan and Curran, 1989).

Fos is a 62 kDa nuclear protein comprised of 380 amino acids. Antibodies used in immunocytochemical studies are generated against subsets of the complete Fos protein amino acid sequence. Antibodies to some peptides of Fos form complexes with both Fos and with Fos-related antigens (Fra). Both Fos and Fra may be induced by a variety of extracellular stimuli and possess common antigenic properties (Sonnenberg et al., 1989a,b; Sharp et al., 1991b). For this reason, the immunoreactivity detected by these antibodies is correctly referred to as Fos-like immunoreactivity (FLI).

III. *c-fos* in the CNS

In neurons of the adult CNS, basal expression of *c-fos* is reported to be very low (Dragunow et al., 1987; Dragunow and Robertson, 1988a) but spontaneous increases are reported to occur in specific brain regions at certain times of the day (Kononen et al., 1990). In general, *c-fos* activation is linked to changes in the rate of metabolic activity in a cell. As shown in Table 3, a variety of stimuli are capable of eliciting a rapid and transient induction of *c-fos* mRNA and protein in neurons (Morgan et al., 1987; Sheng and Greenberg, 1990; Morgan and Curran, 1991).

Table 3. Summary of some of the major stimuli which increase expression of the IEG *c-fos* in specific areas of the mammalian CNS.

Abbreviations are as follows: 4VO: 4 vessel occlusion; s.c.: subcutaneous injection; i.p.: intraperitoneal injection; icv: intracerebroventricular; i.v.: intravenous injection; stx: stereotaxic injection; AMY: amygdala; AOB: accessory olfactory bulb; CP: caudate putamen; CRB: cerebellum; CTX: cortex; HIP: hippocampus; HYP: hypothalamus; LC: locus coeruleus; MOB: main olfactory bulb; MPOA: medial preoptic area; NAc: nucleus accumbens; PVN: paraventricular hypothalamic nucleus; SEP: septum; SCN: suprachiasmatic nucleus; SON: supraoptic nucleus; STM: striatum; ECS: electroconvulsive shock; CRH: corticotropin-releasing hormone; CCK: cholecystokinin

Stimuli	Model	Region CNS	Reference
Neurotransmitters/ Drugs			
yohimbine	Wistar rat, i.p.	LC, PVN	Tsujino et al., 1992
clonidine	Wistar rat, i.p.	PVN, SON	Tsujino et al., 1992
L-DOPA	S-D rat, stx	STM	Robertson et al., 1989
haloperidol	Wistar rat, i.p.	STM	Dragunow et al., 1990
CCK	S-D rat, i.p.	SON, PVN	Verbalis et al., 1991
morphine	S-D rat, s.c.	CP	Chang et al., 1988
amphetamine	S-D rat, i.p.	STM/SEP	Graybiel et al., 1990
cocaine	S-D rat, i.p.	STM/SEP	Graybiel et al., 1990
Hormones			
CRH	i.c.v.	PVN	Andreae & Herbert, 1993
growth hormone	Wistar rat, i.v.	ARCN, PVN	Minami et al., 1992
angiotensin II	S-D rat, i.v.	SFO, OVLT	McKinley et al., 1992
melatonin		SCN	Kilduff et al., 1992
insulin		lateral HYP	Bahjaoui-B., et al., 1994
Seizures			
bicuculline	BD IX rat, i.v.	HIP	Gass et al., 1992
picrotoxin	S-D rat, i.p.	HIP, CTX	Saffen et al., 1988
metrazole	mice, i.p.	HIP, CTX	Dragunow & Robertson, 1987b
ECS	Swiss mice	limbic, HYP	Daval et al., 1989
kindling	S-D rat	CTX, AMY	Dragunow & Robertson, 1987a
EtOH withdrawal	mice	HIP, CTX, CRB	Dave et al., 1990
EAA			
glutamate	Wistar rat, stx	HIP	Kaczmarek et al., 1988
quinolinic acid	Fischer rat, stx	HIP	Walker & Carlock, 1993
NMDA	S-D rat, s.c.	ARCN	MacDonald et al., 1990
Domoic Acid			Robertson et al., 1991
Kainic Acid	Wistar rat, i.p.	HIP, AMY	Popovici et al., 1990
Other Perturbations			
dehydration	S-D rat, i.p.	SON, PVN	Ding et al., 1994
hemorrhage	rat	SFO, OVLT	Badoer et al., 1992
hypoxia-ischemia	rat, 4VO	HIP	Wessel et al., 1991
brain injury	CD1 mice	CTX	Dragunow & Robertson, 1988b
pain, heat	S-D rat	SC-dorsal horn	Williams et al., 1990b
sexual behaviour	S-D rat	MPOA, NAc	Robertson et al., 1991
odour, urine	CD1 mouse	AOB, MOB	Schellinck et al., 1993

This is just a short list of the many stimuli that elicit *c-fos* expression in the CNS. In the interest of brevity, the principal emphasis of this chapter will be on the effect of EAAs and sex steroids on FLI in adult and developing brain. I will also discuss the benefits and drawbacks of using *c-fos* expression as a marker of metabolic activity in the brain.

The Use of *c-fos* as a Marker of Cellular Activation:

A significant body of evidence now exists to support the contention that expression of FLI in the CNS is a reliable indicator of recent cell membrane depolarization. For example, following kindled seizures, FLI appears sequentially in cohorts of neurons known to be recruited during kindling in the cerebral cortex, amygdala, piriform and entorhinal cortex, and hippocampus (Dragunow et al., 1988b; Morgan and Curran, 1991).

Further support for the contention that FLI reflects changes in levels of neural activity is obtained from studies of circadian variation in brain activity. In mammals, daily rhythms such as activity level and body temperature, are controlled by an internal clock or pacemaker. This is influenced by environmental lighting changes but in their absence a cyclical rhythm is maintained with a periodicity that is very close to 24 hrs. (Rusak et al., 1990). The suprachiasmatic nucleus (SCN) is believed to be a primary centre for the control of mammalian circadian rhythms. Kononen et al. (1990) reported that rats maintained on a 12:12 hr. light:dark cycle (lights on 0700 - 1900 hr) exhibited a circadian rhythmicity in basal FLI in various brain areas including the SCN. Density of FLI in the SCN at 2400 hrs. was half that observed at 1200 hrs. and corresponds with the circadian variation in neuronal activity reported by Gillette and Prosser (1988). Earnest et al. (1992) have demonstrated that the endogenous

rhythm in c-fos expression is maintained under conditions of constant light. Photic stimulation during the dark phase of the light-dark cycle dramatically increased c-fos mRNA and FLI in the region of the SCN which receives retinal input (Rusak et al., 1990). It has been recently reported that in animals maintained in constant darkness, light pulses induced FLI in the SCN only during the subjective dark period (Schwartz et al., 1994).

Most of the evidence accumulated to date supports the contention that the induction of FLI is a reliable indicator of neuronal activity. Under some circumstances, however, there may not be a good correlation between FLI and neuronal activity. The absence of FLI should not be interpreted as lack of activity. Some neurons may express IEGs which are not members of the Fos family in response to stimulation, and others which express c-fos in response to some stimuli may fail to do so in response to others. For instance, tonically active tuberoinfundibular dopamine neurons in the ARCN do not stain for Fos but they do express Fra in an activity dependent manner (Hoffman et al., 1994).

The rate of (^{14}C)-2-deoxyglucose (2-DG) uptake has been used to measure focal changes in neural metabolism and several investigators have reported elevated levels of FLI in neurons following stimuli known to increase 2-DG uptake in those cells. For example, direct electrical stimulation of the sensory or motor cortex of rats increased both FLI and 2-DG uptake in cerebellar neurons (Sharp, 1984; Sharp and Ryan, 1984; Sagar et al., 1988; Sharp et al., 1988; Sharp et al., 1989). Patterns of FLI expression and 2-DG uptake do not coincide in all instances. Electrical stimulation of the sensory or motor cortex increased 2-DG uptake in neurons of the caudate putamen without altering FLI (Sagar et al., 1988; Sharp et al., 1989).

In some instances, FLI is detected in the absence of elevated 2-DG uptake. Elevated levels of FLI were observed in neurons of the SON and PVN of rats

subjected to dehydration (water deprivation for 24 hrs.) (Sagar et al., 1988) but mild dehydration (replacement of drinking water with 2% saline for 5 days) does not elevate 2-DG uptake in these cells (Schwartz et al., 1979; Lightman et al., 1982; Gross et al., 1985). Discrepancies between FLI and 2-DG results have also been reported by other investigators (Jorgenson et al., 1989) and may reflect the fact that both neuronal excitation and inhibition may increase regional metabolic activity (Ackermann et al., 1984; Kadekaro et al., 1985), thereby enhancing the regional uptake of 2-DG.

In many cases, the results of c-fos immunocytochemistry mapping studies have proven to be consistent with data obtained from electrophysiological recording, neuroanatomical tract tracing, in situ hybridization, and/or autoradiography experiments. FLI is not an absolute indicator of elevated neuronal activity but when interpreted cautiously, c-fos immunocytochemistry represents a powerful tool for neuroscientific research, especially in neuroendocrinology (Hoffman et al., 1992, 1993).

Excitatory Amino Acids and c-fos

In vitro:

Primary cultures of cerebellar granule cells respond to L-glutamate with a dose-dependent rise in c-fos mRNA followed closely by a rise in Fos protein (Szekely et al., 1989). In these cultures, glutamate-induced c-fos expression is potentiated by glycine (positive allosteric modulator of NMDA receptor) and is blocked by APV, Mg^{2+} and PCP. It appears that the NMDA receptor has an exclusive role in the control of c-fos expression in these cultured neurons because kainate, quisqualate, carbachol (M_2 muscarinic agonist), GABA and baclofen ($GABA_B$ agonist) failed to alter c-fos expression.

Didier et al. (1989) examined the relationship between Fos protein and the neurotrophic effect of NMDA and K^+ on cultures of mouse cerebellar granule cells. They reported that neuronal survival and maturation are enhanced by constant depolarization or activation of the NMDA receptor and are closely associated with c-fos expression. Interestingly, c-fos expression in cerebellar granule cells which are constantly stimulated becomes detectable on and after 6 days of exposure and persists at high levels until the cultures begin to decline. The authors believe that the "long-term expression of the c-fos gene might be related to some aspect of the late differentiation process of cerebellar granule cells" (Didier et al., 1992). In contrast, a 30 second "pulse" of glutamate stimulates a rapid and transient rise in c-fos and other IEG mRNA in primary cultures of neurons from cerebral cortex and striatum (Vaccarino et al., 1992). In addition, c-fos mRNA levels were significantly higher following a 30 second "pulse" than following a 10 minute "pulse" of glutamate. The c-fos expression observed by Vaccarino et al. (1992) appears to be related to cell activation whereas Fos in the cerebellar granule cells (Didier et al., 1992) seems indicative of the demise of the cell culture. The discrepancy in these findings may be related to culture technique, cell type, or animal age or it may be a result of the length of exposure to glutamate.

In vivo:

EAA-induced c-fos expression has been extensively investigated in various animal models (see Table 3). Kaczmarek et al. (1988) demonstrated that a stereotaxic injection of L-glutamate but not saline resulted in a significant increase in c-fos mRNA in the hippocampus. Both intraperitoneal and stereotaxic injections of kainic acid (KA) increase c-fos expression in neurons of limbic structures (Le Gal La Salle, 1988; Popovici et al., 1990; Sonnenberg et al., 1989b).

Smeyne et al (1993) reported that, in *fos-lacZ* transgenic mice, systemic injection of kainic acid induced continuous elevated levels of *c-fos* gene expression in dying neurons. Schreiber et al. (1993a) have reported that, in addition to a transient increase in Fos expression, there is a sustained increase in *c-fos* mRNA in brain regions exhibiting neuronal death following KA-induced seizure activity. The protein synthesis inhibitor, cycloheximide attenuates KA-induced neuronal damage and blocks prolonged *c-fos* expression (Schreiber et al., 1993b).

Agonists of the NMDA receptor, such as NMDA and quinolinic acid (QA), also elicit *c-fos* expression in rodent brain. NMDA agonist delivery by s.c., i.p., i.v. or by stereotaxic injection enhances transcription of the IEG, *c-fos* (Cole et al., 1989, Sonnenberg et al., 1989b; MacDonald et al., 1990, 1993; Aronin et al., 1991; Walker and Carlock, 1993, Massieu et al., 1992; Lee et al., 1993). Activation of the NMDA receptor also increases transcription of AP-1 transcription factors (Aronin et al., 1991) and increases AP-1 binding activity in mammalian CNS (Sonnenberg et al., 1989b).

Calcium appears to be a second messenger coupling activation of NMDA and non-NMDA receptors to the expression of *c-fos* (Lerea et al., 1992). However, the different glutamate receptors seem to have distinct mechanisms for transducing the calcium influx signal (Lerea and McNamara, 1993). For instance, inhibitors of phospholipase A_2 and cyclo-oxygenase blocked only the NMDA-stimulated rise in *c-fos* mRNA and the calmodulin antagonist, calmidazolium, blocked only the KA-regulated rise in *c-fos* mRNA.

Our laboratory has reported that NMDA rapidly and transiently increases *c-fos* mRNA and FLI in the ARCN of immature female rats in an age- and dose-dependent manner (MacDonald et al., 1990, 1993). Fos production is blocked by pre-injection with the NMDA antagonists, MK-801 and APV (MacDonald et al., 1990). Although NMDA treatment of female rats releases LH (most likely via

activation of LHRH neurons) and increases FLI in periventricular organs, it does not increase *c-fos* expression in LHRH neurons (Lee et al., 1993). Saitoh et al. (1991) have confirmed the absence of NMDA-induced FLI in LHRH neurons in their model of hypogonadal mice. However, FLI was detected in CRF neurons of the PVN, noradrenergic neurons of the locus coeruleus and dopaminergic neurons of the MBH (Saitoh et al., 1991). The *c-fos* evidence indicates that other neurotransmitter systems may be involved in NMDA-stimulated LH release perhaps by a direct influence on LHRH nerve terminals in the median eminence.

The complexity of the neural network connecting NMDA stimulation with *c-fos* expression is further elucidated by the studies by Gloria Hoffman's group on NMDA-induced FLI in lactating rats. Abbud et al. (1992) demonstrated that i.v. administration of NMDA increases *c-fos* expression in many brain regions including the hippocampus and the cortex. Interestingly, NMDA-stimulated FLI is still found in the hypothalamus but is absent from the hippocampus and cortex of lactating rats. Removal of the suckling stimulus and/or progesterone blockade (RU-486) resulted in the restoration of the pattern of NMDA-induced FLI observed in cycling adult rats (Abbud et al., 1993).

The NMDA receptor also appears to be involved in the induction of *c-fos* following brain injury and seizures. Herrera and Robertson (1990) reported that pretreatment with the NMDA antagonists, ketamine, MK-801 and PCP, inhibit the rise in FLI observed after minor disruption of the pia mater. Brain-injury induced FLI was not influenced by muscarinic, opioid or calcium channel antagonists (Herrera and Robertson, 1990). Similarly, Dragunow et al. (1990) reported that induction of *c-fos* mRNA and FLI in response to injury of the frontoparietal cortex is completely abolished by ketamine but only weakly inhibited by nifedipine (Ca^{2+} channel antagonist) and trifluoperazine (calmodulin antagonist). Intraventricular injection of the competitive NMDA

receptor antagonist, CPP, blocked c-fos expression following cortical cavity lesion to the brain (Sharp et al., 1990).

NMDA antagonists:

The noncompetitive NMDA antagonist, MK-801, paradoxically, induces FLI in neurons of CNS. Dragunow and Faull (1990) reported the induction of FLI in the neocortex, dorsal and ventral midline thalamic nuclei and central grey, 2 to 24 hrs. after MK-801 injection (i.p.). Lee et al. (1994) have made similar observations in the paraventricular nucleus. MK-801 induction of c-fos mRNA in the cingulate cortex can be blocked by administration of central muscarinic antagonists (atropine) (Hughes et al., 1993b).

c-fos in the Developing and Aging Brain

The spontaneous expression of c-fos during development of the rat CNS has been studied by several research groups. Gonzalez-Martin et al. (1991) reported that in Wistar albino rats, FLI occurs precisely in layer VIb of the cerebral cortex between embryonic day 20 (E20) and P1. The authors suggest that c-fos expression may be related to acquisition of a mature phenotype, synaptogenesis, action of nerve growth factor (NGF) or cell death. Elevated levels of c-fos mRNA were detected using Northern analysis in rat brain (whole brain, cerebellum, cerebral cortex) during the time of most rapid growth (E16 to P13), period of increased myelinization and synaptogenesis (P13 to P30), and functional synaptic activity (P13 to P60) (Gubits et al., 1988). In the rat, elevated levels of FLI are seen in the cerebral cortex, striatum, hippocampus and cerebellum at specific times during the first 2 weeks of postnatal life in a sequence that closely parallels ontogenic development (Alcantara and Greenough, 1993).

The stimulatory effect of seizure activity on *c-fos* expression is well known in the adult rat brain (Krukoff, 1994). However, the neonatal brain appears to have a differential IEG response to seizure induction. For instance, Schreiber et al. (1992) reported little evidence of *c-fos* induction following kainic acid-induced seizures in rats younger than 13 days. Interestingly, Jensen et al. (1993) have reported a variation in the staining pattern and time course of FLI in juvenile (P10) vs. adult rats following pentylenetetrazol- (PTZ) and flurothyl-induced convulsions. Similar patterns of FLI in juvenile and adult animals were observed in amygdala, pyriform cortex and hypothalamus whereas the most obvious differences were in the cortex.

Despite the variation in the IEG response to seizure activity, neonatal rat brains appear to respond to ischemia and nociception with enhanced *c-fos* expression in a similar manner to adults. For instance, Munell et al. (1994) reported that hypoxia-ischemia induced *c-fos* mRNA in various brain areas of 7 day old rat pups. In addition, FLI was observed in lumbar spinal cord neurons of 1-3 day old rats following injection of capsaicin or formalin into, or application of mustard oil to, a hindpaw (Williams et al., 1990a,b).

Kitraki et al. (1993) have also reported an age-related decrease in spontaneous *c-fos* expression in cerebral cortex and cerebellum using Northern analysis. In contrast to this, Yang and Koistinaho (1990) reported an increase in FLI in sympathetic neurons of the superior cervical ganglion in 26 vs. 2 month old rats. As in the young rats, differential *c-fos* expression in the aged rat brain probably reflects changing neuronal activity with age.

Estrogen and *c-fos*::

Molecular cloning and cell transfection techniques have allowed researchers to identify and map segments of DNA that are able to bind the

estrogen receptor and stimulate gene transcription. Specifically, Weisz and Rosales (1990) have localized an estrogen response element (ERE) 1060 to 1300 bases upstream of the startsite of transcription of the c-fos gene and Hyder et al. (1992) have found another ERE in the untranslated 3' flanking region of the murine c-fos proto-oncogene. These studies suggest the possibility that c-fos gene transcription can be directly enhanced by the interaction of the estrogen-receptor complex with the ERE associated with c-fos. Although the estradiol-receptor complex may bind directly to the regulatory domains of various genes with growth-promoting effects, it is also conceivable that intermediate genomic events, such as the activation of c-fos, may mediate some of the organizational effects of estradiol.

Estrogen treatment is known to stimulate c-fos expression in uterus (Weisz and Bresciani, 1988; Loose-Mitchell et al., 1988, Gibbs et al., 1990b; Papa et al., 1991), cervicovaginal tract (Scrocchi and Jones, 1991), anterior pituitary gland (Szijan et al., 1992) and in a human breast cancer cell line (Wilding et al., 1988). Other proto-oncogenes such as c-jun, c-myc, jun-B and jun-D are also induced by estrogen in uterus (Weisz et al., 1990; Webb et al., 1993; Bigsby and Li, 1994, Nephew et al., 1994) and anterior pituitary (Szijan et al., 1992). The estrogen antagonist, tamoxifen, also stimulates c-fos and jun-B expression in differential layers of rat uterine tissue (Nephew et al., 1993).

There is some controversy in the literature regarding steroid-induced expression of c-fos in the CNS. Insel (1990) reported that 12 to 48 hrs. after EB (100 μg/kg), ovariectomized adult rats increased the number of cells exhibiting FLI in the anterior medial preoptic area (AMPOA), MPOA, medial amygdala nucleus and VMN of the hypothalamus. Using a slotblot technique, Cattaneo and Maggi (1990) discovered a biphasic increase in c-fos mRNA in the midbrain (includes POA) 30 min. and 24 hrs. after estrogen (estradiol (E_2): 375 mg/kg)

treatment. In contrast, Gibbs et al. (1990a,b) did not detect changes in c-fos mRNA or FLI up to 6 or 24 hrs., respectively, post-treatment (E$_2$: 5 mg or EB: 5 mg) of adult, prepubertal (P25) or perinatal (P0 or P10) rats. Several explanations may account for the divergent reports of estrogen activation of c-fos in brain including a 10 to 20-fold difference in the dose of steroid and variations in the time course used by each group.

An interesting observation is that c-fos message and FLI appear to peak 24 and 24 to 48 hrs. after estrogen treatment, respectively (Insel, 1990; Cattaneo and Maggi, 1990). This is inconsistent with the rapid and transient induction following other physiological stimuli. For instance, FLI peaks in the hypothalamus 1.5-2 hrs after injection of NMDA (MacDonald et al., 1990, 1993). The time course of steroid-induced FLI in uterus also appears slower than observed with other stimuli. Gibbs et al. (1990b) reported that in uterus, c-fos mRNA increases 2-fold 3 hrs. post-treatment and FLI is present at 3 hrs and peaks at 6 hrs. post-treatment. Szijan et al. (1992) reported that c-fos mRNA in uterus increases significantly by 30 min., peaks at 1 hr. and remains above controls through the 16 hr. study period after estrogen administration. Although the reported time course is variable, estrogen induction of c-fos is relatively slow, especially in brain tissue, and it is unlikely that c-fos is induced directly by estrogen. It is more feasible that c-fos expression may be initiated secondarily by another estrogen-inducible peptide (Insel, 1990).

LHRH Neurons and c-fos:

Steroid induction of FLI has been localized to LHRH neurons of the POA and anterior hypothalamus of mouse, rat, hamster and ewe (Hoffman et al., 1990; Lee et al., 1990a,b, 1992b, Moenter et al., 1993; Wu et al., 1992; Doan and Urbanski, 1994; Berriman et al., 1992). Administration of estradiol followed 24

hrs. later by progesterone (procedure used routinely to induce ovulation) activated *c-fos* in LHRH neurons of prepubertal female but not male rats. (Hoffman et al., 1990). The proto-oncogene product, Jun, is also expressed by LHRH neurons on the afternoon of proestrus and through the morning of estrus (Lee et al., 1992a). Since both proto-oncogene products, Fos and Jun, are expressed by LHRH neurons during proestrus the Fos-Jun heterodimers may intercalate with the DNA and alter gene expression during the LH surge. In the LHRH neurons, steroid induction of Fos and Jun appears to be indirect and may require the combination of both gonadal steroids: estrogen and progesterone. The LH surge arises due to estrogen positive feedback on the hypothalamus and although LHRH neurons do not have estrogen receptors (Shivers et al., 1983), estrogen may alter LHRH neuronal activity and *c-fos/c-jun* activity secondarily via steroid-concentrating neurons impinging on the network of LHRH neurons.

There appears to be a strong correlation between *c-fos* induction and increased LH secretion (Lee et al., 1992b). Ovariectomized rats given only estrogen replacement rather than estrogen followed by progesterone had lower LH concentrations and a correspondingly smaller number of FLI-positive LHRH neurons (Lee et al., 1990a). FLI is not detected in LHRH neurons during diestrus 1, 2 or estrus but is expressed in 40% of LHRH neurons on the afternoon of proestrus and the number of FLI-positive LHRH neurons increases relative to plasma LH concentrations (Lee et al., 1992b). Increased *c-fos* expression in LHRH neurons of the ewe during the LH surge has also been reported (Moenter et al., 1993). The LH surge and the number of LHRH neurons expressing Fos is reduced by administration of the progesterone antagonist RU-486 (Lee et al., 1990a). Central administration of interleukin-1β suppresses LHRH release as well as the proestrus-associated increase in *c-fos* expression (Rivest and Rivier, 1993). Food deprivation which is known to block ovulation and pulsatile LH

secretion also reduces *c-fos* expression in LHRH neurons of the Syrian hamster (Berriman et al., 1992). Increased expression of *c-fos* in LHRH neurons may be related to the recruitment of newly activated LHRH neurons and induction of the preovulatory LH surge (Lee et al. 1992b; Hoffman et al., 1990). Cesnjaj et al. (1993) have reported LHRH-induced *c-fos* expression in a line of immortal LHRH neurons (GT1-7 cells) and hypothesize that the proestrus rise in FLI-positive LHRH neurons may be related to a positive feedback action of LHRH on its own receptors (ultra-short feedback loop). Lloyd et al. (1994) have similarly shown that the diminution in the LH surge observed in middle-aged rats (Cooper et al., 1980; Wise, 1982) is paralleled by a decrease in the percentage of Fos- and Jun-positive LHRH neurons.

FLI in LHRH neurons may not always correlate with an increase in secretory activity. In the Syrian hamster, the preovulatory LH surge occurs 3 hrs. prior to the increase in *c-fos* expression in LHRH neurons (Doan and Urbanski, 1994). The latent production of Fos in LHRH neurons may represent the activation of a mechanism arresting the preovulatory surge of hormones or perhaps the activation of a synthetic pathway which is replacing depleted LHRH stores following the surge (Doan and Urbanski, 1994).

c-Fos Expression in Two Models of Precocious Puberty

The immunocytochemical localization of the *c-fos* protein product can be used cautiously to localize neuronal activation in the CNS. In this chapter I have mapped the site of action of NMDA and MSG in our two models of precocious puberty (i.e. neonatal MSG treatment, prepubertal NMDA treatment) by identifying FLI in sections through the CNS. Our laboratory has published two articles in this area (MacDonald et al., 1990; 1993) and the experiments in this chapter represent a continuation of this line of research. We specifically wanted to know if NMDA is acting in other areas besides the ARCN and whether FLI changes during the course of treatment in either model of puberty.

Material and Methods

I. Animals

The source, housing and maintenance of the Sprague-Dawley rats used in these experiments is identical to that described in the Material and Methods section of Chapter 2.

II. Drugs

In addition to the drugs described in Chapter 2, 17-β-estradiol-3-benzoate (EB; E-9000) was obtained commercially from Sigma Chemical Company (St. Louis, MO). MK-801 was generously provided by Merck Sharp and Dohme Research Labs (Rahway, NJ).

III. Immunocytochemistry for Fos-Like Immunoreactivity (FLI)

Rats were deeply anaesthetized using Somnotol (P2-P9=0.1 ml; P10-P21=0.2 ml; >P21=0.3 or 0.4 ml; i.p.) and perfused transcardially with Dulbecco's phosphate buffered saline (DPBS; pH 7.4; 4°C) followed by 4% paraformaldehyde (PFA; pH 7.4; 4°C) in 0.1 M phosphate buffer (PB). The volume of DPBS and fixative used in the perfusions varied depending on the age of the rat (P2 to P15 = 30 ml; P21 to 35 = 60 ml; >P35 = 90 ml). Following the perfusion, the brains were carefully removed and placed in 4% PFA (4°C) for approximately 1 week before sectioning.

To examine FLI in the ARCN, SFO, anterior medial POA (AMPOA) at the level of the OVLT, paraventricular hypothalamic nucleus (PVN) and SON of juvenile female rats, the brains were blocked by one coronal cut 3 mm rostral to the optic chiasm and another cut 1 mm caudal to the mammillary bodies. The block of brain was glued (Crazy Glue) to an aluminum stage, immersed in cold DPBS and sectioned at 50 μm using a Vibratome (Series 1000). To examine the

AP, the brains were blocked and transferred to 15% sucrose overnight. The following day they were sectioned at 40 μm using a cryostat microtome (-23°C). The neonatal rat brains (d2 to d9) were blocked by one coronal cut 2 mm rostral to the optic chiasm and another 1 mm caudal to the mammillary bodies. The brains were glued to a stage, immersed in cold buffer and all areas of the brain (including AP) were sectioned at 100 μm using a Vibratome. The sections were transferred to mesh bottomed baskets in 24 well tissue culture plates (see Fig. 26) and placed on an oscillating table at room temperature throughout the entire immunohistochemical procedure unless otherwise noted. The tissue was washed three times (10 min. per wash) with 0.01 M PBS and 0.2% Triton X-100 (TX), followed by another wash (20 min.) in 0.3% hydrogen peroxide in PBS and TX for 20 min. to remove endogenous peroxidase activity in the sections. Next, the sections were washed twice (10 min. per wash) with PBS and TX and subsequently incubated in 3% normal rabbit serum (NRS; Gibco Laboratories, NY) and 0.1% sodium azide in 0.01 M PBS to block non-specific binding by the primary (1°) antibody. After the blocking step, the sections were incubated for 5 days at 4°C with a sheep polyclonal antibody to fos oncoproteins (Cambridge Research Biochemicals, Cheshire, U.K.; OA-11-823) and was used at a dilution of 1:20,000 (v:v) in 3% NRS and 0.1% sodium azide in 0.01 M PBS.. To prevent evaporation of the 1° antibody solution, the tissue culture plates were wrapped in parafilm and placed in a plastic bag containing damp paper towels. After the incubation, the sections were washed three times (10 min. per wash) in PBS and TX and incubated in a biotinylated rabbit anti-sheep antibody (Vector Laboratories; BA 6000) at a dilution of 1:500 in 1% NRS and 1% TX at room temperature for 1 hr. After washing the sections 3 times in PBS and TX, they were incubated with avidin-biotin-peroxidase complex (Vector laboratories; PK 4000) at a dilution of 1:200 (5 ml of solution A and B/ml) in 0.01 M PBS. at room

temperature for 1 hr. The sections were rinsed three times (10 min. per wash) in PBS and TX and then placed in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma; D-5905) and 0.6% ammonium nickel (II) sulfate hexahydrate (Aldrich; 33,485-5) in 50 mM Tris buffer (TB) for 5 min. Hydrogen peroxide at a concentration of 0.006% was added to each well. Once sufficient blue-black reaction product was visible in the sections (10-15 min.), the reaction was stopped by rinsing three more times in PBS and TX. The brain slices were mounted on subbed glass microscope slides, dried overnight, dehydrated in a series of 50% to 100% ethanol washes, cleared in xylene and coverslipped with Entellan.

We have previously shown that omission of the 1° antibody or pre-adsorption of the 1° antibody with Fos peptide (2 mg/ml; 16 amino acids; Cambridge Res. Biochemicals, UK, cat. # OP-11-3210) eliminated FLI (results not shown).

Figure 26. Apparatus used in the immunocytochemistry experiments.

(a) Photograph of 24 well tissue culture plate used in the immunocytochemistry protocol. (b) Diagram of the apparatus used for tissue incubation during the immunocytochemical staining process for Fos and other antigens. Two tissue culture plates (as shown in (a)), one intact and one with the bottom of each well removed, are stacked on top of the other. Mesh-bottomed sample cups are placed into the bottomless wells of the uppermost plate. Buffers and antibody solutions are placed in the intact tissue culture plate on the bottom plate. Individual sections are placed in the sample cups and are moved between solutions by removing the upper plate with sample cups and placing into fresh solution. Note that this simple apparatus reduces damage to tissue by minimizing manipulation of sections.

A

B

Lid

24 well plate
(bottom removed)

sample cup

24 well plate
(intact)

section

mesh

PBS or antibody
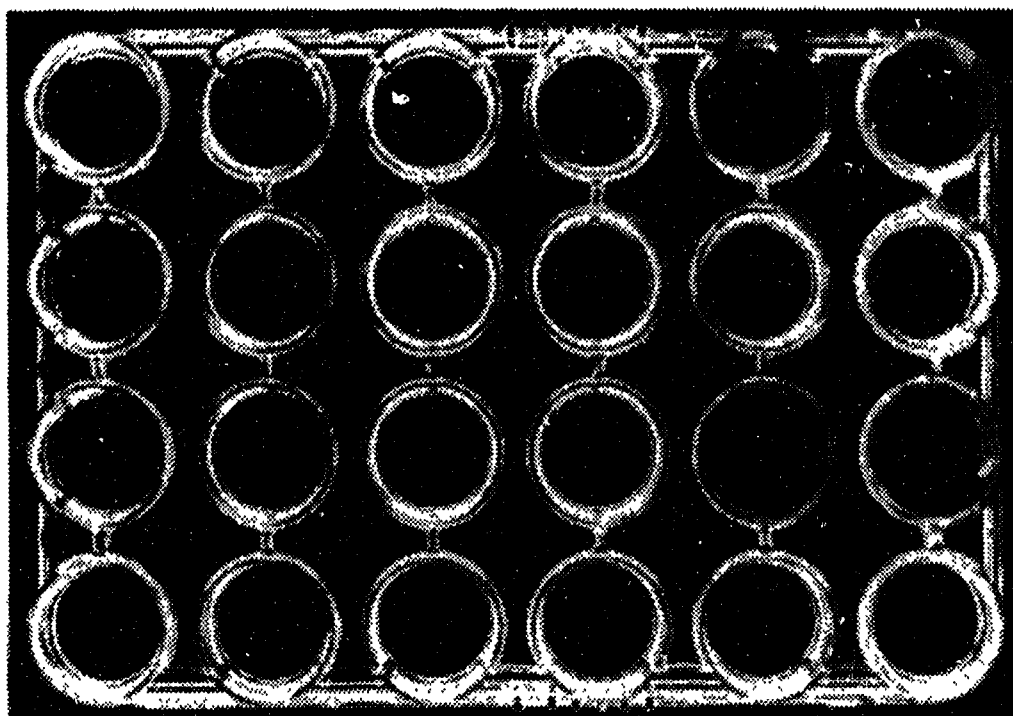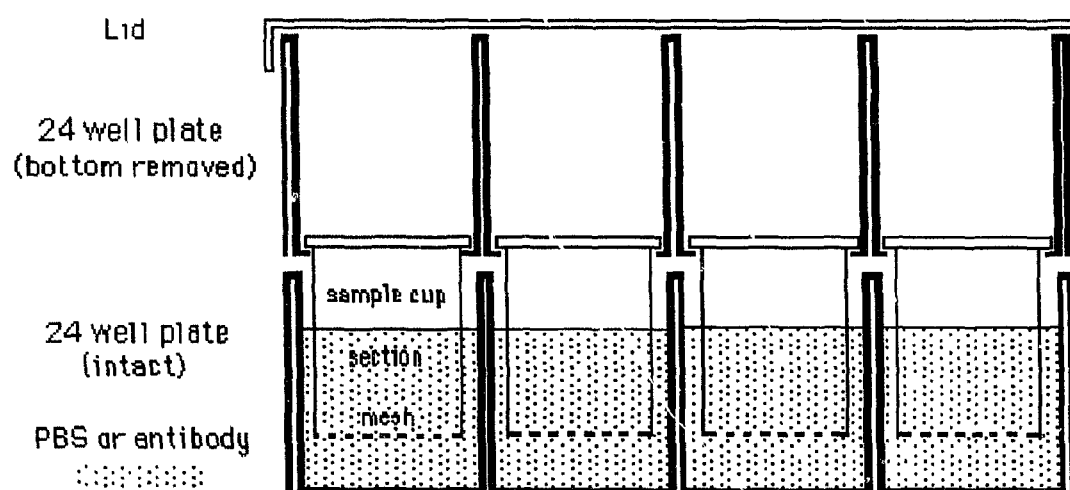
Fig. 26

IV. Experiments with Neonatal Rats

(1) The Effect of MSG on FLI in the CNS of Neonatal Female Rats

Female rats were injected with 0.9% saline (0.1 ml; s.c.;) or MSG (2 or 4 mg/g; s.c.) on the morning of P2 (n=3-6/group). Two hrs. post-treatment, the rat pups were deeply anaesthetized and perfused. The brains were prepared and sectioned as described previously. Coronal sections through the AMPOA, SFO, SON, PVN, ARCN, and AP were processed for FLI.

(2) c-fos expression in MBH with increasing doses of MSG

Litters of female rats were injected with 0.9% saline vehicle (0.1 ml; s.c.) or with various doses of MSG (0.25, 1.0, 2.0 or 4.0 mg/g; s.c.) on the morning of P2 (n=4/group). Two hrs. post-injection, the pups were anaesthetized and perfused. The brains were prepared and sectioned as described previously and coronal sections through the entire MBH were processed for FLI.

(3) The Effect of MK-801 on MSG- and NMDA-Induced FLI in MBH

Neonatal (P2) female rat pups were injected with NMDA (3 mg/kg; s.c.) or MSG (2 or 4 mg/g; s.c.) or MK-801 (1.0 mg/kg; s.c.) (n=3/group). To test if MK-801 could block MSG- and NMDA-induced FLI rats (n=3/group) were co-injected with either MSG (2 or 4 mg/g; s.c.) and MK-801 (1.0 mg/kg; s.c.), or NMDA (3.0 mg/kg; s.c.) and MK-801 (1.0 mg/kg). Two hours after treatment, the rats were anaesthetized and perfused. and brains removed for sectioning. 75 to 100 μm sections through the entire MBH were processed for FLI.

(4) The Effect of Sequential MSG Injections on FLI in MBH of Neonatal Rats

A standard method of disrupting the reproductive and endocrine systems of rats is to administer MSG (4 mg/g; s.c.) to neonates at 2, 4, 6 and 8 days of age. We examined the pattern of FLI in the MBH over the course of this treatment and compared it to neonatal rats receiving the drug for the first time.

(i) Several litters of female rats were injected with saline (0.1 ml; s.c.) or MSG (4 mg/g; s.c.) at 2 days of age. On postnatal day 4 (P4), half of the control and MSG-treated group from P2 were injected with saline (0.1 ml; s.c.) and the other half of each group were injected with MSG (4 mg/g; s.c.). The rats in the four groups (Treatment on P2/P4:   sal/sal, MSG/sal, sal/MSG and MSG/MSG; n=6/group) were anaesthetized, perfused and later examined for FLI in coronal sections (75 to 100 µm) of the MBH.

(ii)   Several litters of female rats were injected with saline (0.1 ml; s.c.) or MSG (4 mg/g; s.c.) at 2, 4 and 6 days of age. On postnatal day 8 (P8), half of the control and MSG-treated group from P2 to P6 were injected with saline (0.1 ml; s.c.) and the other half of each group were injected with MSG (4 mg/g; s.c.). The rats in the four groups (Treatment on P2/P4/P6/P8:   sal/sal/sal/sal, MSG/MSG/MSG/sal, sal/sal/sal/MSG, and MSG/MSG/MSG/MSG; n=6/group) were anaesthetized, perfused and later examined for FLI in coronal sections of the MBH.

V. Experiments with Juvenile Rats

(1) NMA Induced FLI in the CNS of Juvenile Female Rats

Female rats were injected with NMA (30 mg/kg; s.c.; n=3/group) or 0.9% saline (0.1 ml; s.c.; n=3/group) at 1100h on P28. At 1300 h, the rats were anaesthetized, perfused and the brains removed. Representative coronal sections (50 μm) through the AMPOA, SFO, SON, ARCN and AP were processed for FLI.

(2) MK-801 Blockade of NMDA-Induced FLI in the MBH

Female rats were injected with NMDA (20 mg/kg; s.c.; n=3) or MK-801 (0.1 or 1.0 mg/kg; n=2/dose) between 1000 and 1100 h on P28. To block NMDA-induced FLI, two groups of rats were injected with one of two doses of MK-801 (0.1 or 1.0 mg/kg; s.c.; n=2/group) 30 min. prior to an injection of NMDA (20 mg/kg; s.c.). Two hrs. after the treatment, the rats were deeply anaesthetized, perfused and the brains removed for sectioning. Six representative coronal sections (50 μm) of the MBH of each animal were processed for FLI.

(3) The Effect of Sex Steroids on NMDA-Induced FLI in the MBH

A group of female rats were ovariectomized at 23 days of age. Immediately after the surgery and every other day thereafter, half the group were treated with oil vehicle (0.1 ml; s.c.; n=3) and the other half with EB (10 μg; s.c.;n=3). On P28, the rats were injected with NMDA (20 mg/kg; s.c.) and 2 hrs. later were deeply anaesthetized and perfused. The brains were carefully removed, post-fixed in 4% PFA and sectioned. Six representative coronal sections (50 μm) of the MBH of each animal were processed for FLI.

(4) FLI in the MBH of Female Rats Administered Multiple Injections of NMDA

We compared the effect of a single NMDA injection with multiple NMDA injections on FLI in the MBH of young female rats. From 24 to 27 days of age, one group (NMDA (x1); n=3) of rats were administered 0.9% saline (0.1 ml; s.c.) and at 28 days of age they received a single injection of NMDA (20 mg/kg; s.c.) at 1100 h. A second group (NMDA (x5); n=3) received daily injections of NMDA (20 mg/kg; s.c.) from 24 to 28 days of age at 1100 h. Both groups were deeply anaesthetized and perfused 2 hrs. after the day 28 NMDA injection (1300 h). Alternate serial sections through the MBH of all animals were processed for FLI (approx. 12-14 sections/animal).

Results

I. Experiments with Neonatal Rats

(1) The Effect of MSG on FLI in the CNS of Neonatal Female Rats

We examined FLI in the cortex, hippocampus and circumventricular areas of the brain of P2 female rats, 2 hrs. after treatment with MSG at a dose which elicits precocious sexual maturation (2 mg/g). Dense nuclear FLI was found in the cortex (Fig. 27C), AMPOA at the level of the OVLT (arrowhead; Fig. 27A), SON (not shown), PVN (not shown), SFO (Fig. 27B), AP (Fig. 27D) and MBH (Fig. 28C). Dark bands of FLI, resembling barrels were evident in layers 2/3 of the granular retrosplenial cortex (RC) of the pups following MSG treatment (see arrowhead; Fig. 27C). The pattern of FLI in the MBH was a semi-circular band of dense FLI with relatively few immunopositive cells between the semi-circle and the ventral surface of the brain (see arrowheads, Fig. 28C). FLI was almost completely absent in the saline-treated control pups (Fig. 27E to 27H).

Figure 27. - FLI in neonatal female rats following MSG treatment.

FLI in various neuronal areas 2 hrs. following MSG (2 mg/g; s.c.; <u>left</u> of figure; 4 mg/g not shown) or 0.9% saline (0.1 ml; s.c.; <u>right</u> of figure) treatment of neonatal female rats on the morning of P2 (n=3 to 6/group) (scale bar = 100 μm). <u>A & E</u>: AMPOA at OVLT; <u>B & F</u>: SFO; <u>C & G</u>: retrosplenial cortex; <u>D & H</u>: AP. Note the dense immunostaining following MSG treatment compared to low basal levels evident in saline-treated controls.

D

MSG P2   Saline P2
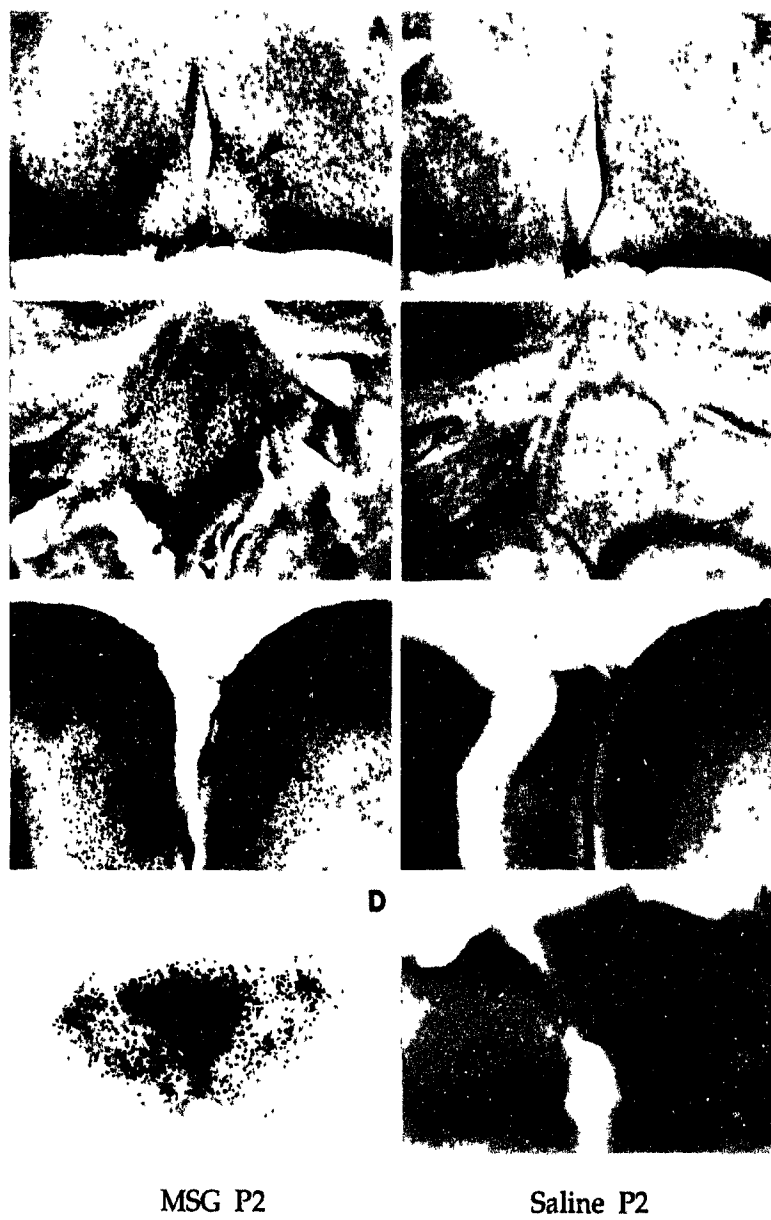
Fig. 27

(2) c-fos Expression in MBH with Increasing Doses of MSG

FLI in the ARCN/ME area was examined 2 hrs. after treatment of P2 female rat pups with 0.25, 1, 2 or 4 mg/g of MSG. All doses of MSG induced FLI in the area of the ARCN/ME. FLI after the lowest dose of MSG (0.25 mg/g) was restricted to the arcuate area immediately adjacent to the ventral portion of the third ventricle (see arrowhead, Fig. 28A). The radius of the semi-circular staining pattern seems to enlarge with the rising dose of MSG (see arrowheads Fig. 28C). Note, however, that the semi-circular band of FLI always lay beneath the ventromedial nucleus (VMN) of the hypothalamus (see *). FLI was sparse in the MBH of saline-treated control pups (not shown). With increasing doses of MSG, the background staining in the ependymal cells lining the third ventricle becomes lighter (see arrow Fig. 28C and D).

Figure 28. - Dose response of FLI in the ARCN of neonatal pups after MSG. Photomicrographs of FLI in the ARCN of neonatal female rat pups 2 hrs. after treatment with increasing doses of MSG (A: 0.25 mg/g; B: 1.0 mg/g; C: 2.0 mg/g; D: 4.0 mg/g) on the morning of P2 (n=4/group). Note semi-circular pattern of FLI in the ARCN appears to increase in size with increasing doses of MSG. (Scale bar = 100 μm).

0.25 mg/g   2.0 mg/g

1.0 mg/g   4.0 mg/g
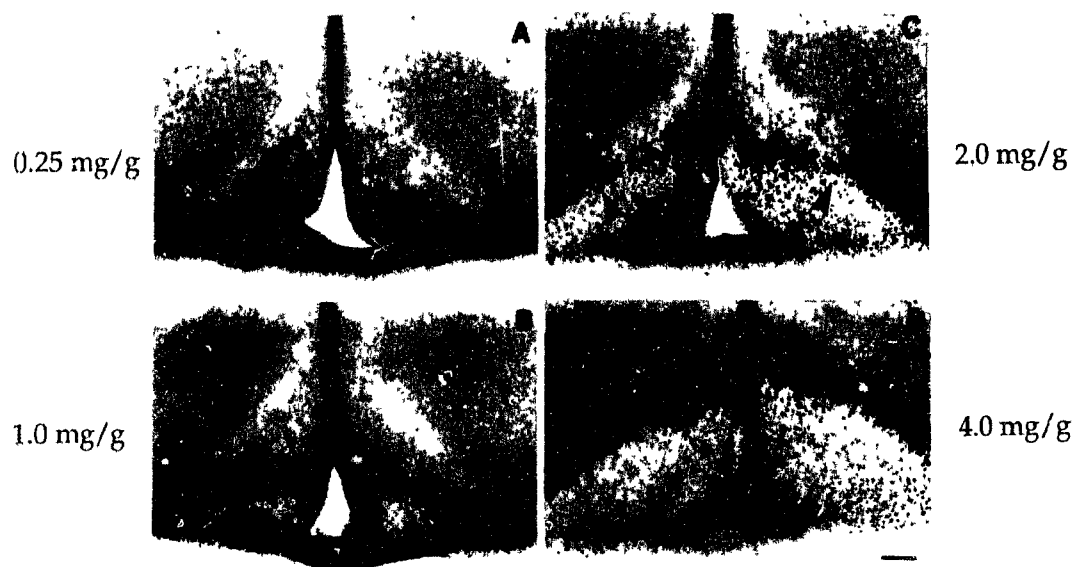
Fig. 28

(3) The Effect of MK-801 on MSG- and NMDA-Induced FLI in the MBH

In this experiment we examined whether the noncompetitive NMDA receptor antagonist, MK-801, could block the effect of neonatal MSG or NMDA on FLI in the MBH of neonatal pups. Both MSG (4 mg/g) and NMDA (3 mg/kg) treatment increase FLI in the MBH of P2 female rat pups (Fig. 29A and C) but the staining pattern is very different. As seen previously, MSG-treatment induces a dense arc of fos-positive cells below the VMN (see arrowhead), overlying a clear and possibly edematous ARCN (see star; Fig. 29C). Following NMDA, very dark staining is observed in the ARCN and the dorsomedial aspect of the VMN, forming almost parallel lines of FLI on each side of the ventricle at 45 degrees (see arrowheads Fig. 29A). MK-801 treatment (1 mg/kg) alone did not induce FLI in the MBH of neonatal rat pups (not shown). Co-injection with MK-801 markedly inhibited NMDA-induced FLI in the ARCN and only a small number of fos-positive cells were evident immediately adjacent to the third ventricle and ME (see arrowhead, Fig. 29B). However, co-injection with MK-801 had little or no effect on MSG-induced FLI (Fig. 29D). MK-801 treatment may possibly reduce the radius of the arc (see arrowheads Fig. 29D) of FLI but this has not been confirmed with quantitative analysis.

Figure 29. - NMDA and MSG-induced FLI in the ARCN of P2 female rats and the effects of pretreatment with MK-801.

Photomicrographs of FLI in the ARCN of P2 female rats 2 hrs. after treatment with (A): NMDA (3 mg/kg; s.c.); (B): NMDA and MK-801 (3 mg/kg and 1.0 mg/kg, respectively; s.c.); (C): MSG (4 mg/g; s.c.); and (D): MSG and MK-801 (4 mg/g and 1.0 mg/kg, respectively; s.c.) (n=3/group) (scale bar = 100 μm).

NMDA

MSG

MK-801
+ NMDA

MK-801
+ MSG
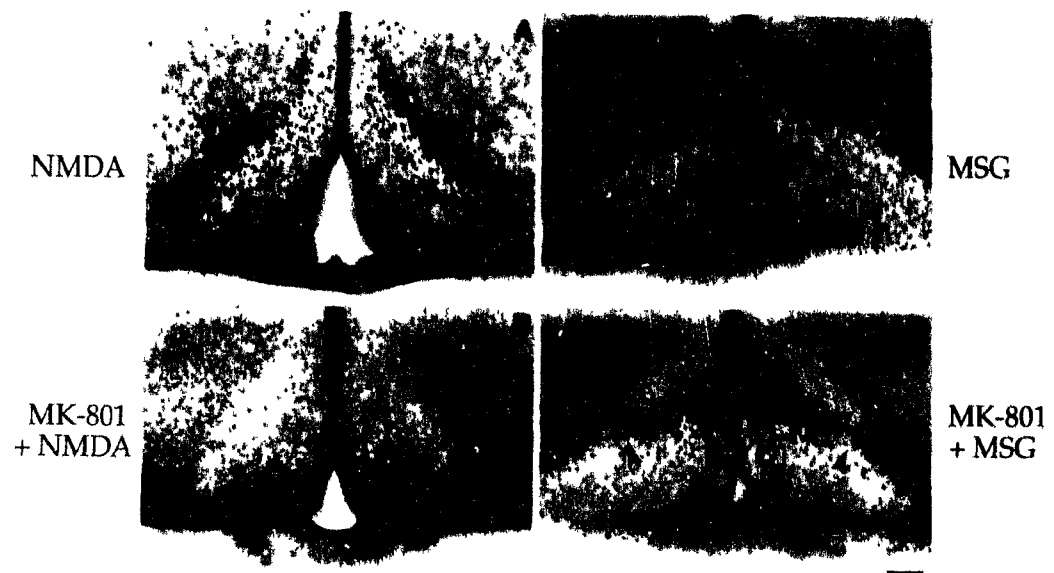
Fig. 29

(4) The Effect of Sequential MSG Injections on FLI in the MBH of Neonatal Rats

We studied the changing pattern of MSG-induced FLI in neonatal rats between the ages of P2 and P8. We also examined whether FLI was altered by previous alternate daily injections of MSG from P2 (4 mg/g MSG on P2, P4, P6 and P8; experimental paradigm used to cause extensive neuronal damage and endocrinopathies). The extent of MSG-induced FLI decreases with age (see arrowheads showing decrease in radius of arc of fos; Fig. 30A vs. 30D). On P4, the rats receiving MSG for the first time have a different staining pattern than those receiving MSG for the second time (Fig. 30A vs. 30B) as in the latter group there appears to be a new population of cells adjacent to the third ventricle responding to MSG with Fos production (see arrowhead; Fig. 30B). Rat pups receiving their fourth injection of MSG on P8 express very little Fos (see arrows; Fig. 30E) compared to age-matched rats receiving MSG for the first time (Fig. 30D). In the MSG (x4) group, the third ventricle appears to be enlarged and the median eminence appeared longer and thinner than controls (see star, Fig. 30E). Very few cells exhibiting FLI are evident in the MBH of control saline-treated rats at either age (Fig. 30C and 30F).

Figure 30. - MSG-induced FLI in ARCN of P2 female rats following MSG from P2 to P8.

FLI in the ARCN of juvenile female rats 2 hrs. after MSG treatment (4 mg/g; s.c.) on P4 (left of figure) or P8 (right of figure) in animals receiving their first, second or fourth injection (n=6/group). (A): 4 mg/g MSG on P4 only; (B) 4 mg/g MSG on P2 and P4; (C): saline (0.1 ml; s.c.) treatment on P4; (D) 4 mg/g MSG on P8 only; (E) 4 mg/g MSG on P2, P4, P6 and P8; (F) saline treatment on P8 (scale bar = 100 μm).

MSG
x 1

MSG
x 2 or x 4

Saline

P4   P8
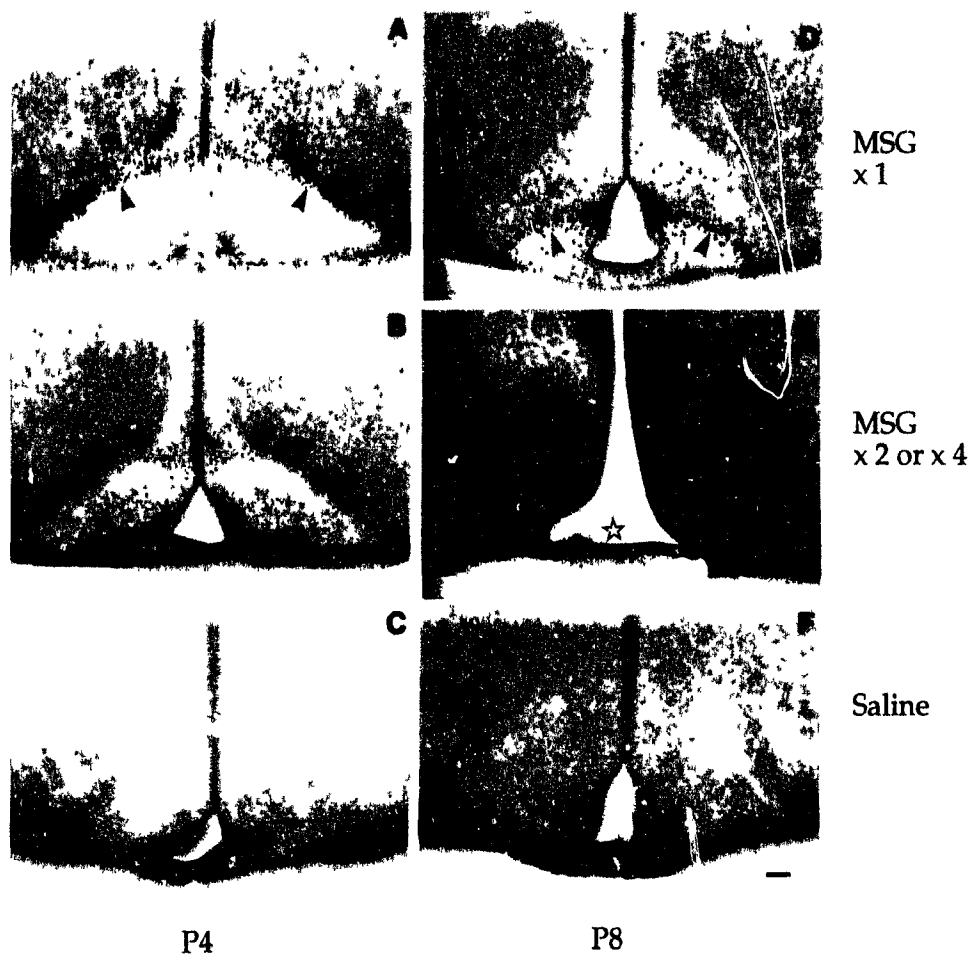
Fig. 30

II. Experiments with Juvenile Rats

(1) NMDA-Induced FLI in the CNS of Juvenile Female Rats

Juvenile female rats express fos-like immunoreactivity (FLI) in several neuronal areas 2 hrs. after a subcutaneous injection of NMDA. FLI following NMDA has been localized to the AMPOA at the level of the OVLT, SFO, SON, PVN, ARCN/ME, and AP (see Fig. 31). FLI was not observed in the SCN or the hippocampus (not shown). Saline treated rats had low level FLI expression in comparable neuronal locations (not shown).

(2) MK-801 Blockade of NMDA-Induced FLI in the MBH

NMDA treatment caused a significant induction of FLI in the mediobasal hypothalamus of prepubertal female rats (Fig. 32A). Treatment with the noncompetitive antagonist, MK-801 (0.1 or 1.0 mg/kg), had no effect on FLI in the ARCN/ME area (Fig. 32D). However, pre-treatment (30 min.) with MK-801 either partially (0.1 mg/kg; Fig 32C) or completely (1.0 mg/kg; Fig. 32B) suppressed NMDA-induced FLI in the ARCN/ME area 2 hrs. after treatment.

Figure 31. - Photomicrograph of FLI in the CNS of juvenile female rats following NMDA.

FLI in coronal sections (50 μm) of various areas of the CNS of P28 female rats 2 hrs. after treatment with NMDA (20 mg/kg; s.c.; A to F) or saline (not shown) (n=3/group). A = AMPOA at the level of the OVLT; B = PVN; C = AP; D: SFO; E: SON; F: ARCN/ME. Scale bar = 100 μm.
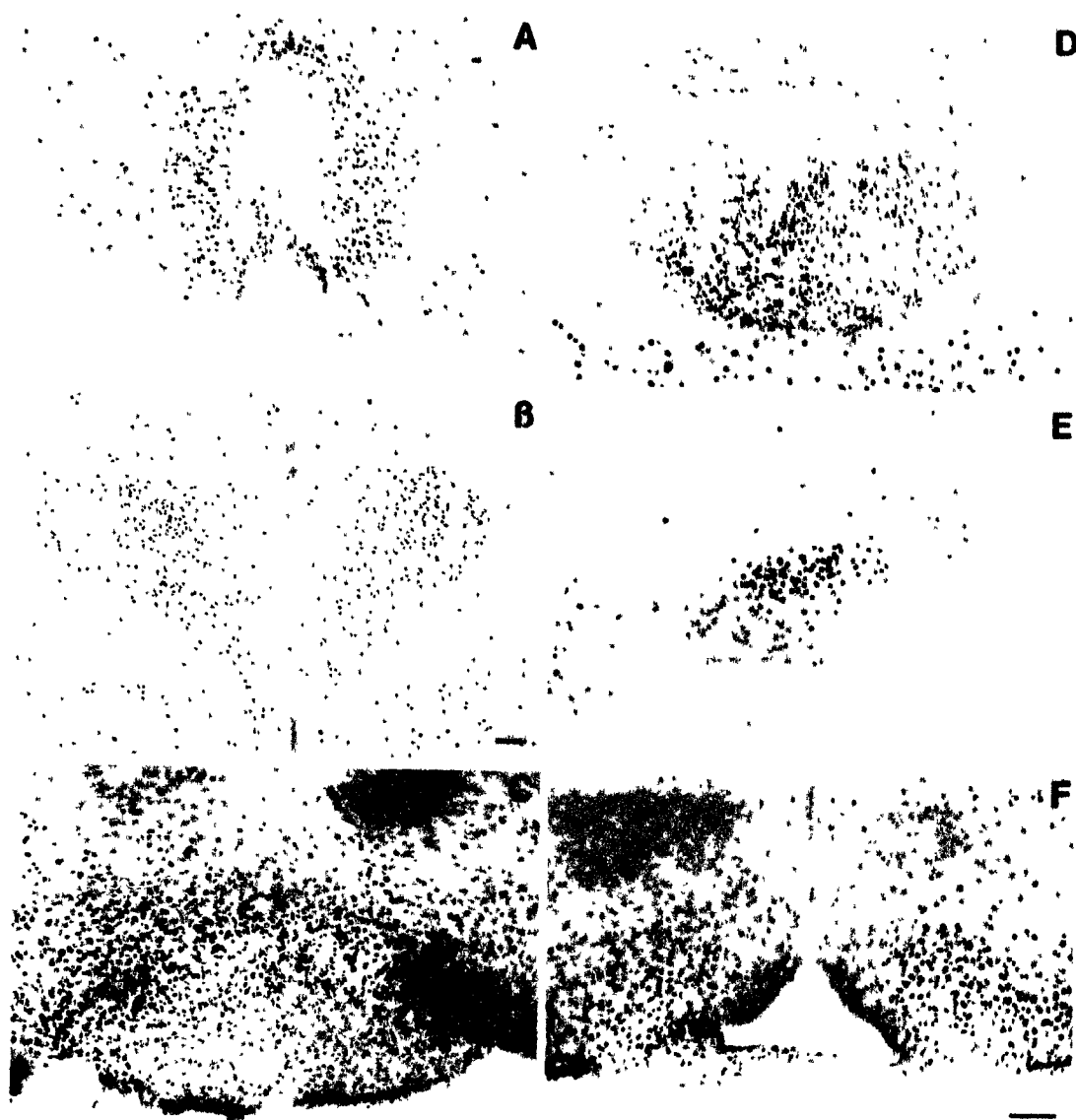
Fig. 31

Figure 32. - Photomicrograph of MK-801 blockade of NMDA-Induced FLI in the ARCN.

FLI in coronal sections (50 μm) through the ARCN of P28 female rats treated (s.c.; 0.1 ml; 2 hrs. prior) with NMDA (20 mg/kg), MK-801 (0.1 and 1.0 mg/kg) or one of two doses of MK-801 (0.1 or 1.0 mg/kg) 30 min. prior to an injection of NMDA (20 mg/kg) (n=2-3/group). A = NMDA (20 mg/kg); B = MK-801 (1.0) + NMDA; C = MK-801 (0.1) + NMDA; D = MK-801 (1.0 mg/kg). Scale bar = 100 μm.
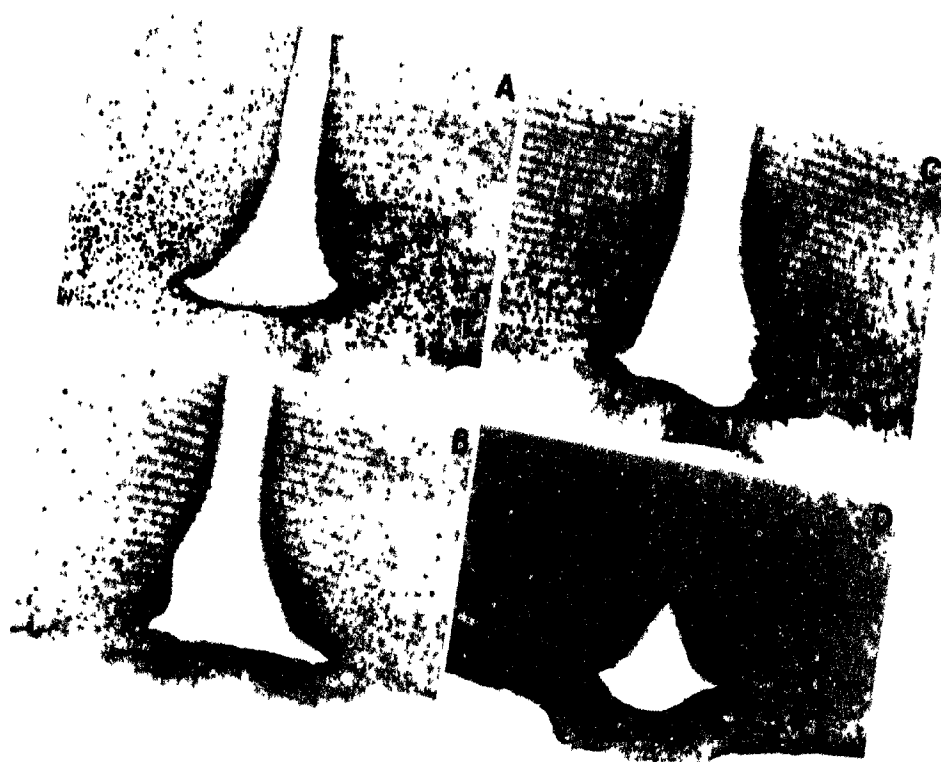
Fig. 32

(3) The Effect of Sex Steroids on NMDA-Induced FLI in the MBH

We compared FLI in the ARCN of ovariectomized oil-treated and ovariectomized estrogen-replaced prepubertal female rats 2 hrs. after treatment with NMDA. Animals in both treatment groups responded to NMDA with the same pattern and level of *c-fos* expression in the ARCN (not shown).

(4) FLI in the ARCN of Female Rats Administered Several Injections of NMDA

We compared the level and pattern of NMDA-induced FLI in the ARCN of prepubertal female rats receiving NMDA for the first time (NMDA (x1)) and those receiving it for the fifth time (NMDA (x5)) (as per our NMDA model of early puberty). Figure 33 illustrates the differences in FLI in sections from the rostral (Fig. 33A vs. D), medial (Fig. 33B vs. E) and caudal (Fig. 33C vs. F) areas of the ARCN, 2 hrs. after treatment in the NMDA (x5) vs. NMDA (x1) group. In all animals examined, there was a distinctive decrease in the intensity of FLI in the ARCN/ME of the NMDA (x5) group (Fig. 33A, B and C) compared to the NMDA (1) group (Fig. 33D, E and F). This decrease in FLI was apparent in all tissue sections from rostral to caudal portions of the arcuate nucleus.

Figure 33. - FLI in the ARCN of P28 female rats after treatment with NMDA for the first or the fifth time.

Groups of female rats were injected with NMDA or saline once daily at 1100 hrs. from P24 to P27 and on P28 both groups received an injection of NMDA at 1100 hrs (n=3/group) (x1 NMDA on right; x5 NMDA on left of figure). Two hours post-treatment the rats were anaesthetized, perfused and brains prepared for sectioning. Sections from different levels of the ARCN were examined for FLI: A,D: rostral; B,E: medial; C,F: caudal. (scale bar = 100 μm).

X5   X1
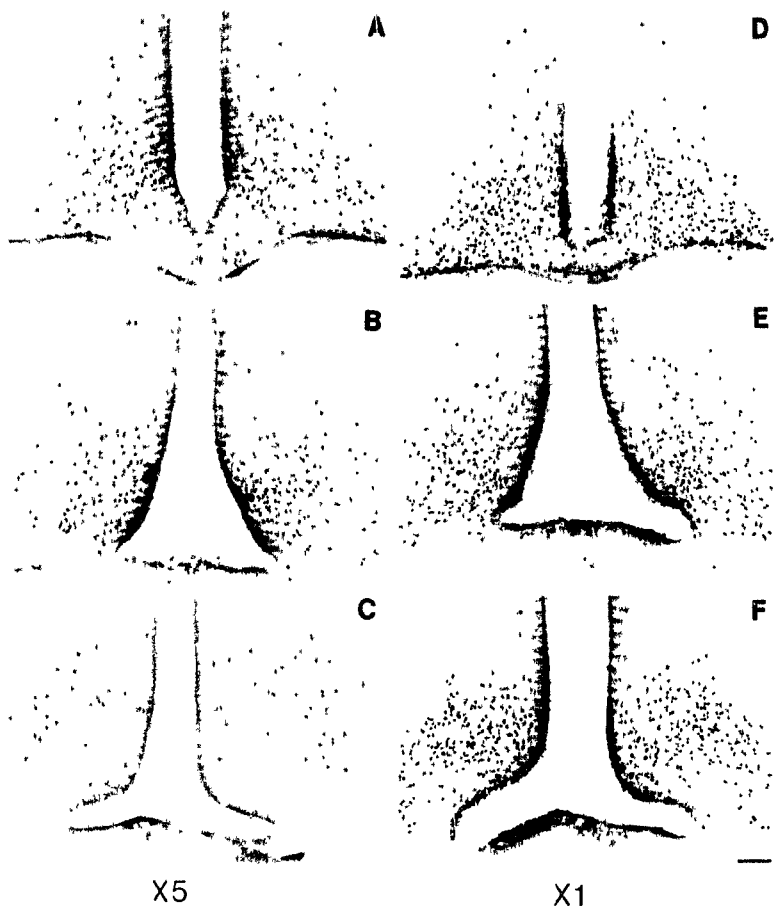
Fig. 33

Discussion

As discussed in Chapter 2, and previously reported by MacDonald and Wilkinson (1990), MSG treatment of neonatal rats and/or NMDA treatment of juvenile female rats, significantly accelerates the onset of first ovulation. Several hypotheses have been generated to explain these phenomena, including the removal of an inhibition or increased excitation, perhaps via synaptogenesis. However, our first step in determining the mechanism of MSG- and NMDA-stimulated precocious puberty was to localize their sites of action in the CNS using FLI as a marker of neuronal activation.

I. Neonatal Rats

The neonatal hypothalamus expresses *c-fos* in response to glutamate receptor stimulation. MSG treatment induces FLI in circumventricular organs (CVOs) (ARCN/ME, SFO, AP, OVLT), SON, PVN and the retrosplenial cortex (RC) of neonatal (P2) female rats. FLI is principally localized to areas of the CNS which, in adults, lack a BBB (i.e. the CVOs) despite lacking a mature barrier at this neonatal age (for review see Himwich, 1962). Our results are in accordance with the work of Olney (1969, 1971) and Seress (1982) who localized the neurotoxic effects of glutamate treatment. High dose MSG treatment used in neuroendocrine ablation destroys all ME neurons (Olney, 1969) and 80 to 90% of the neurons in the ARCN (Olney, 1971). Olney and Price (1978) have stated that glutamate treatment severely damages the AP and SFO and minimally damages the SCO and OVLT but these results have not been confirmed by other researchers. The ARCN/ME may be more susceptible to MSG than other circumventricular nuclei due to the presence of high concentration of glutamate receptors in this region (Monyer et al., 1992).

The localization of FLI suggests that MSG may be acting at several locations to initiate precocious sexual maturation. Many of the areas affected by MSG treatment have been shown to have physiological roles in reproductive function. LHRH neuronal soma have been localized to the AMPOA and their nerve terminals to the ARCN/ME area (Ibata et al., 1979; Witkin et al., 1982). Electrical stimulation of the SFO induces LH secretion (Donevan et al., 1989).

Unfortunately, the absence of FLI does not negate neuronal activation. MSG may act on other neuronal sites that do not increase c-fos expression accordingly (see earlier section on "The use of c-fos as a Metabolic Tract Tracer"). For instance, Kubo et al. (1993) reported that neonatal MSG treatment destroys hippocampal CA1 neurons but we did not detect an increase in FLI in the hippocampus following MSG treatment of neonates. However, in our experiments, the presence of dense "barrels" of FLI in layers 2/3 and relatively dark FLI in layer 5 of the granular RC may represent stimulation of the hippocampus. Wyss and Van Groen (1992) have mapped the connections between the hippocampal formation and the RC and have reported that the granular RC receives dense projections from CA1 and the subiculum. The pattern of c-fos expression in the RC may be related to trans-synaptic neuronal activation via the topographically organized connections between the RC and the hippocampal formation (CA1).

We focused on the ARCN in our c-fos experiments because of its well known susceptibility to MSG treatment. In our model, glutamate increases FLI in a dose-dependent manner in the MBH of P2 female rats. The clear, hemi-circle of tissue beneath the band of FLI in the ARCN that we observed 2 hrs. post-MSG treatment (see Fig. 27, 28) is similar to that reported by Bruni et al. (1991) in the domoic acid (DA)-treated mouse. In that article, DA-injured areas were clearly distinguished by pallor and vacuolated neuropil due to the swelling of dendrites,

astrocytes and neurons. Illustrated in Bruni et al. (1991) is a well-delineated area of cell damage and swelling, radiating outward away from the ME, OVLT and AP within 30 minutes of EAA treatment. The lesion in the mouse hypothalamus included the entire ARCN and extended upward to the boundary of the fibrous capsule of the VMN. The suggestion by Bruni et al. (1991) that the damaging effects of DA appeared to 'radiate' from the structures lacking a BBB is clearly illustrated in our dose-response analysis of MSG-induced FLI in the VMH. The arc of FLI and the clear area beneath seems to enlarge with increasing concentrations of MSG (0.25 to 4 mg/g). The effects of glutamate appear to be scaled along a diffusion gradient leading away from the ME and may explain the absence of FLI in the ventral ARCN. For instance, a high concentration of MSG in proximity to the ME may arrest transcription and/or translation processes preventing c-fos expression. In contrast, a lower concentration of MSG at a greater distance from the ventricle may stimulate neuronal activation leading to c-fos expression. This is certainly observed in focal models of cerebral ischemia where neuronal damage radiates outward from a central location towards the penumbra and protein synthesis, but not transcription, is arrested in the most central locations (Kinouchi et al., 1993). C-fos mRNA mapping studies using in situ hybridization histochemistry (ISHH) would compliment FLI data and help to determine localized transcription and/or translation blocks following neonatal MSG treatment. In addition, the arc of FLI following MSG does not seem to correspond to any anatomical structure (Paxinos and Watson, 1986) and may simply be related to the moving front of a stimulatory concentration of glutamate.

The pattern of MSG-induced FLI in the MBH changes significantly over the course of treatment used to induce gross endocrinopathies (4 mg/g on P2, P4, P6 and P8) to the point where the only fos-positive cells appear to be localized to

a small area immediately adjacent to the corner of the third ventricle (see Fig. 30E). This staining pattern is preserved in the peripubertal animal treated neonatally with MSG as NMDA-induced FLI in P28 female rats is significantly reduced and displaced ventrally (see MacDonald et al., 1990). Interestingly, 2 mg/g of MSG on P2 which causes early puberty does not appear to change the pattern of NMDA-induced FLI in the peripubertal rat. This implicates other nuclei or perhaps more subtle changes in the ultrastructure of the neuronal network in the events leading to early sexual maturation.

Similar findings to ours were reported by Meister et al. (1989) using the high dose MSG regimen. They examined MSG-treated rats at 1 month of age and found significant damage to the ventrolateral, ventromedial, and to some extent the dorsomedial division of the ARCN. They noted that the remaining cells in the DM division were condensed and both DM ARCN and VMN had been displaced ventrally (see Fig. 34 redrawn from Meister et al., 1989). The ventral displacement of the VMN appears to be dose dependent and the architecture is conserved in the adult animal (Seress, 1982).

Despite the massive changes in the architecture of the ARCN that we see following repetitive MSG treatment (as viewed by the differential *c-fos* response to NMDA in the ARCN), both control and MSG-lesioned animals release the same amount of LH in response to NMDA as juveniles (MacDonald and Wilkinson, 1992). Perhaps the stimulation of acute LH release is not related to the mechanism leading to precocious puberty.
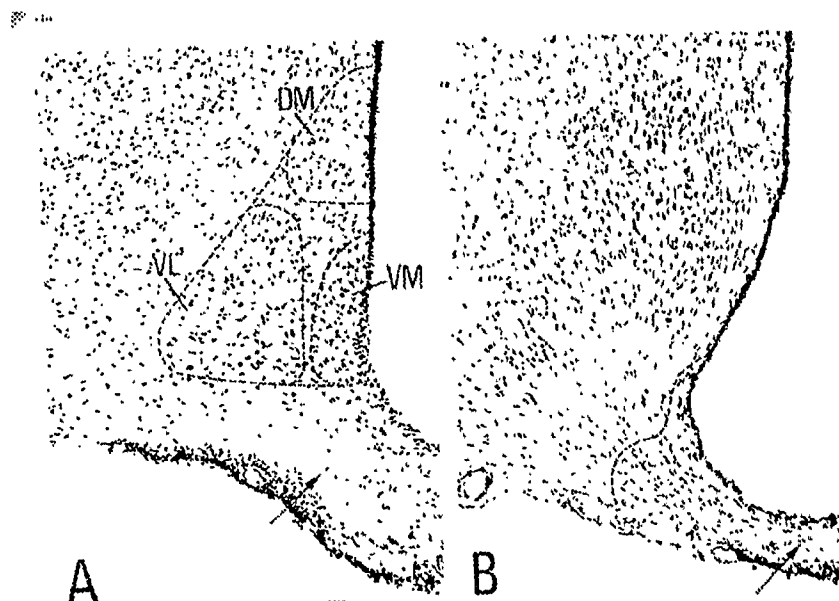
Figure 34. - Photomicrographs of the ARCN of saline- and MSG-treated female rats.

Cresyl-violet stained sections of the arcuate nucleus of control (A) and MSG-treated (B) rats. Comparison of A with B shows a marked degeneration of neuronal cell bodies in the three main parts of the arcuate nucleus (outlined with broken line) after neonatal administration of MSG. Arrows point at areas in the median eminence essentially devoid of cell bodies. Note marked dislocation of the remaining arcuate nucleus in ventral direction (B). Bar indicates 50 μm. Abbreviations: VL: ventrolateral; VM: ventromedial; DM: dorsomedial. (reprinted from Meister et al., 1989).

NMDA and MSG treatment of neonates induces distinct patterns of FLI in the MBH. For instance, NMDA-treated neonates have a characteristic 'triangular' staining pattern involving the ARCN and ventral portion of the VMN (see Fig. 29), whereas MSG treated rats typically display an 'arc' of fos-positive cells below the VMN. Divergent results were also obtained when the noncompetitive antagonist, MK-801, was co-injected with NMDA and MSG. Interestingly, MK-801 almost completely abolished NMDA-induced c-fos expression but had little or no effect on MSG-induced FLI. The lack of 'anatomical' overlap in FLI and the inability of MK-801 to block MSG-induced FLI was not expected as our laboratory has previously reported that NMDA-sensitive neurons appear to be critical to the induction of MSG-stimulated early puberty (MacDonald and Wilkinson, 1990); i.e. MK-801 blocked the ability of neonatal MSG to induce precocious puberty. However, NMDA (3 mg/kg; s.c.) treatment of neonates (P2) does not stimulate precocious puberty (Natarajan et al., 1994). To fully evaluate this model, glutamate antagonists such as AP-5 (competitive NMDA antagonist), DNQX, CNQX (non-NMDA receptor antagonists) and AF-3 (metabotropic antagonist) should be examined for inhibition of MSG-induced FLI and precocious puberty.

The data suggest that other glutamate receptors may be involved in MSG-induced precocious puberty. Binding studies and autoradiography have confirmed the presence of each major glutamate subtype receptor in all hypothalamic and preoptic regions with the following relative densities: NMDA > metabotropic > kainate >= AMPA (Meeker et al., 1994). The technique of cloning has led to an ex losion of research related to the structure and function of glutamate receptors (for review see Hollmann and Heinemann, 1994) and many reports have emerged illustrating the differential expression of the various subtypes of glutamate receptors and even splice variants during development of

the CNS (Bettler et al., 1990; Pellegrini-Gampietro et al., 1991; Sato et al., 1993; Monyer et al., 1994; Laurie and Seeburg, 1994; Sheng et al., 1994). Condorelli et al. (1992) reported that mGlu.R mRNA (metabotropic receptor) is expressed at constant high levels in the hypothalamus between 1 and 30 days of age but this conflicts with Sortino et al. (1991) who found a peak in the activity of metabotropic receptors at P8 which declined to undetectable levels by P32. Della Vedova et al. (1994) found that the ratio of two splice variants of the NMDA receptor ($NR1_{1xx}$, $NR1_{0xx}$) remains relatively constant through development but there appears to be a peak in $NR1_{1xx}$ between P15 and P18 in the hypothalamus. The developmental expression of kainate or AMPA receptor mRNA has not been examined in the hypothalamus. Despite the lack of data on the developmental expression of glutamate receptors in the hypothalamus, there is reason to believe that other receptors, besides NMDA, may be involved in the MSG-induced acceleration of sexual maturation.

II. Peripubertal Rats

The distribution of NMDA-stimulated *c-fos* expression in the CNS of peripubertal (P28) female rats, with the exception of the RC, is identical to that observed in the neonate. NMA treatment has also been reported to increase FLI in the hippocampus (Lee et al., 1993) and pyriform cortex (Abbud et al.,1993) of adult female rats. We did not observe an increase in hippocampal or pyriform cortex FLI. These differing results may be explained by differences in the age of the rats, dose of drug or mode of administration (i.v. vs. s.c.). For example, Lee et al. (1993) and Abbud et al. (1993) routinely used multiple i.v. injections of NMDA (40 mg/kg). MK-801 treatment inhibited NMDA-induced FLI in the ARCN/ME of juvenile female rats indicating that NMDA is acting through its own glutamate receptor to increase expression of the IEG, *c-fos*.

NMDA increases FLI in the ARCN of juvenile female rats and possibly acts on the neuronal network impinging on LHRH nerve terminals. NMDA.R1 message has been localized to the hypothalamus of male Syrian hamsters and adult rats (Urbanski et al., 1994; Watanabe et al., 1993). Preliminary research in our laboratory has localized NMDA.R1 protein to the ARCN and VMN of juvenile female rats. Watanabe et al. (1993) examined the distribution of NMDA receptor subunit mRNAs in rat forebrain and found NMDA.R1 mRNA throughout the hypothalamus with extremely high levels in the VMN. This is an interesting observation as NMDA treatmment does not appear to increase c-fos expression in the VMN. The BBB may prevent the penetration of this nucleus with NMDA.

NMDA treatment of juvenile rats also increases FLI at the level of the OVLT and may indicate stimulation of the neuronal network impinging on LHRH neuronal soma. NMDA treatment of adult rats does not increase c-fos expression in LHRH neurons (Lee et al., 1993) but it does rapidly trigger a rise in LHRH mRNA (Petersen et al., 1992) and LH release (MacDonald and Wilkinson, 1990). This is puzzling as Urbanski et al. (1994) have recently shown that the line of immortal LHRH cells, GT1-1 and GT1-7, contains NMDA.R1 mRNA. In addition, NMDA increases LHRH pulse amplitude in cultured GT1-1 neuronal cells in a dose dependent manner (Mahachoklertwattana et al., 1994). Goldsmith et al. (1994) have demonstrated that the LHRH neuronal network receives synaptic contacts from glutamate-immunoreactive axons in the OVLT, medial septum and ARCN of the monkey. NMDA may indeed stimulate LHRH neurons directly without increasing c-fos expression. Other EAA may stimulate FLI in LHRH neurons as there appears to be more non-NMDA than NMDA receptor binding in the rostral hypothalamus and POA (Meeker et al., 1994).

NMDA-induced FLI in the peripubertal rat does not appear to be influenced by estrogen status as illustrated by the matching density and distribution of c-fos staining in the ARCN of ovariectomized and estrogen-replaced rats. This is in accordance with the recent publication by Brann et al. (1993b) who showed that estrogen treatment did not alter NMDA receptor binding or NMDA receptor mRNA levels in the hypothalamus of adult rats. Once again, the effects of NMDA on FLI in the ARCN are incongruent with its physiological effects on LH secretion. MacDonald and Wilkinson (1992) compared the percent increase of NMDA- vs. saline-induced LH secretion in OVX, OVX-EB replaced and intact rats and reported that estradiol may potentiate NMDA receptor activation and increase LH secretion in the peripubertal rat. A similar finding has been reported in the sheep and monkey (Estienne et al., 1990; Reyes et al., 1990, 1991). Based on the LH findings, one would hypothesize that FLI may be higher following NMDA stimulation of OVX-EB replaced vs. OVX female rats but this was not the case. It is plausible that FLI may be altered by estrogen status in other areas of the brain such as the AMPOA. In addition, immunocytochemical techniques are not useful for the detection of subtle differences in gene expression. Alternatively, the expression of c-fos in the ARCN may not be related to the control of LH secretion.

Peripubertal female rats previously treated with NMDA express less c-fos than age-matched controls receiving NMDA for the first time. Previous daily NMDA treatment may cause down-regulation of the NMDA receptor as seen by Oster and Schramm (1993) in cultured cerebellar granule cells. The decrease in the c-fos response to NMDA is in accordance with our data on the decrease in LH secretion after repetitive daily injections of NMDA and our hypothesis on the decrease in NMDA.R sensitivity with the onset of puberty.

III. Future Research

Virtually the same periventricular neuronal structures appear to be acted upon by MSG in the neonate and NMDA in the juvenile rat. Several questions and lines of experimentation arise from this information.

(i) What type of cells are sensitive to NMDA treatment? These questions may be answered by using double antibody staining to distinguish neurons, glia and specific neuronal phenotypes (i.e. GABA, dopamine (DA), NA, etc.).

(ii) Do the cells that express c-fos have NMDA receptors or have they been transsynaptically activated? The NMDA receptor has been cloned (Moriyoshi et al., 1991) and very recently, a monoclonal antibody to the R1 subunit of the NMDA receptor has become available commercially (Pharmingen, San Diego, CA, Cat. #: 60021A; Siegel et al., 1994). To answer this question, localization of NMDA receptor expression by in situ hybridization for NMDA.R1 mRNA or an antibody to the NMDA.R1 protein could be used in conjunction with the ICC or ISHH localization of c-fos expression.

(iii) What neuronal nuclei are involved in NMDA- and MSG-stimulated puberty and is the expression of c-fos an integral part of the molecular cascade leading to precocious sexual maturation? Chiasson et al. (1992) have reported that intrastriatal infusion of antisense oligonucleotides to c-fos reduces the amphetamine-induced rise in FLI. They state that "the direct infusion of antisense oligonucleotides permits discrete, reversible inactivation of ger :s in a highly selective manner ..." (Chiasson et al., 1992). The infusion of antisense c-fos into the ARCN, AMPOA or SFO following NMDA or MSG treatment would be a powerful method of examining the role of the different nuclei and the role of c-fos expression in the onset of early sexual maturation.

An interesting observation made by Smeyne et al. (1993) is that c-fos expression can also be an indicator of cell death. In their line of transgenic mice,

fos-lacZ staining is seen in dying neurons in the hippocampus several days after KA treatment and in cells undergoing terminal differentiation (tooth, ovary). This report raises the interesting possibility that FLI in our glutamate models of precocious puberty may be indicative of cell injury or death rather than activation. Are brain cells damaged by our EAA protocols of accelerated sexual maturity? Is damage and repair part of the mechanism through which glutamate and NMDA receptors control the onset of puberty? These questions will be dealt with in the following chapter.

CHAPTER 4 - INDUCTION OF *HSPs* BY EAA

Introduction

All living organisms, from yeast to plants and mammals, activate a similar genetic program in response to adverse changes in their environment; protein synthesis is almost completely arrested except for the expression of a highly conserved family of proteins called heat shock proteins (HSPs) (Lindquist, 1986; Craig, 1989). Ritossa (1962) was the first to demonstrate the rapid initiation of this genetic program following a heat shock but many other metabolic insults such as, sodium arsenite, ethanol, sulfhydryl reagents, hydrogen peroxide, transition series metals, Ca^{2+} ionophores, heavy metals, and amino acid analogues, provoke the same response (reviewed in Lindquist and Craig, 1988; Welch, 1992). As a result of their multi-factorial activation, many researchers refer to the expressed molecules as 'stress proteins'. There is a considerable amount of circumstantial evidence which supports the belief that the function of these proteins is to protect cells from the ill effects of stress (Hahn and Li, 1990). Several laboratories are using the expression of different HSPs as markers of cell stress, much the same way that *c-fos* expression is used as a marker of cell activation.

The study of HSP expression is of interest to us for the purpose of answering the question: are the excitatory amino acid agonists and antagonists used in our models of puberty damaging cells in the brain? As discussed in Chapter 1, it is well recognized that treatment of neonatal rats with MSG causes extensive lesions of the hypothalamus (Olney, 1971). However, very sparse information exists on the neurotoxic effects of low-dose MSG treatment of neonates or NMDA treatment of peripubertal female rats. The chapter devoted to *c-fos* was useful for the localization of neuronal activation after EAA treatment

but gave little information on those cells adversely affected by the drugs. The immunocytochemical identification of HSP in the hypothalamus should demarcate those cells being 'stressed' by the EAA injections.

This introduction will briefly review the literature on the structure and function of stress proteins. Emphasis will be placed on the expression of HSP 70 in the mammalian nervous system.

HSP Structure and Function

The stress proteins are one of the most conserved groups of proteins characterized to date (Lindquist and Craig, 1988). Some members of the bacterial HSP family share as much as 50% homology with their human counterpart and amongst eukaryotes, stress proteins are between 50 and 98% identical (Craig et al., 1993). Due to discrepancies in reporting and to variation in the number and size of the stress proteins found in various organisms, the nomenclature in the HSP literature is very confusing. To further confound the issue, some HSPs have also been shown to be essential under normal growth conditions (Lindquist, 1986; Lindquist and Craig, 1988). These genes, which are very similar to heat shock genes, are expressed constitutively and have been termed heat shock cognate (HSC) genes (Becker and Craig, 1994). Despite the problems with nomenclature, the different heat shock proteins (stress-inducible and cognate) have been organized into families based on molecular weight and sequence homology. The major HSP families include HSP20, HSP70 and HSP90. Other stress proteins exist in the molecular weight range of 8 (ubiquitin), 10, 60 and 110 kDa.

HSPs are directly involved in the process of protein synthesis. The stress molecules play important roles stabilizing the structure of partially folded proteins and participating in the translocation of proteins across the

mitochondrial and endoplasmic reticulum membranes. Due to their involvement in these processes, they have been termed "molecular chaperones" (Ellis and van der Vies, 1991; Ellis et al., 1989). In the following sections I will summarize the structural and biochemical features of HSPs as well as their role in protein synthesis. I will focus on the HSP70 family with relatively brief synopses of HSP20 and HSP90.

HSP 70 Family

The most abundant and best characterized of the stress proteins are members of the HSP 70 family. In mammalian cells there are 3 forms of HSP 70 referred to on the basis of their apparent size on SD^C-polyacrylamide gel electrophoresis: HSP 72, HSP 73 and glucose-regulated peptide (GRP78) which is also known as immunoglobulin binding protein (BiP). There also appears to be a mitochondrial form of HSP70 which is known as Ssc1p in *Saccharomyces cereviscae* (Craig et al., 1989) and is also known as mtp70 in mammals (Leustek et al., 1989). All of the related HSP70s share the common properties of binding ATP (Welch and Feramisco, 1985) and proteins, particularly those that are unfolded (Becker and Craig, 1994). The ATP binding activity resides in an N-terminal fragment (~ 40 kDa) and the ability to bind peptides resides in the C-terminal portion (~25 kDa) of the protein. HSP72 and 73 can be localized to the nucleus and cytoplasm of a cell. The expression of HSP72 is increased in a variety of stressful and nonstressful conditions which are summarized in Figure 35 (Morimoto and Milarski, 1990).
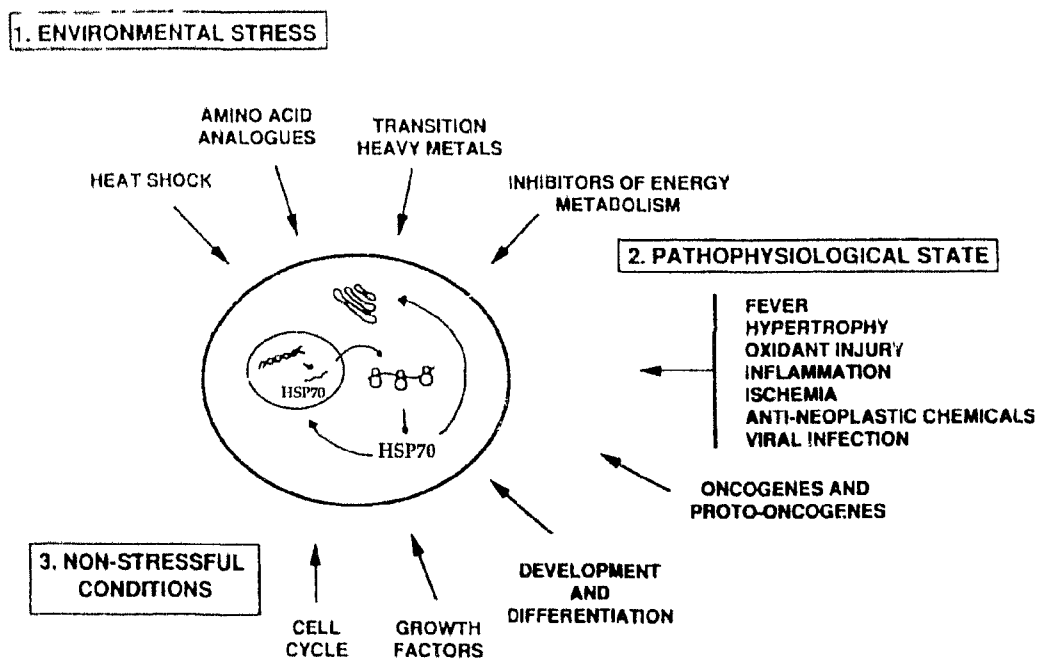
Figure 35. - Summary of the various conditions that have been shown to induce HSP70 expression in the CNS.
(Reprinted from Morimoto and Milarski, 1990).

HSP72 plays a role in regulating the folding of proteins at the ribosome and transporting proteins across subcellular membranes. In contrast, HSP73 is expressed constitutively and responds only slightly to the regulatory conditions affecting the inducible proteins. HSP73 binds to the exposed loop of the clathrin light chain and assists in uncoating clathrin-coated vesicles (Sharp et al., 1993). GRP78 resides in the lumen of the endoplasmic reticulum (ER) and its synthesis is stimulated by the accumulation of misfolded proteins inside this structure (Kozutsumi et al., 1988; Normington et al., 1989; Rose et al., 1989). Mtp70 appears to be necessary for the import of proteins from the cytoplasm to the mitochondrial matrix (Kang et al., 1990; Osterman et al., 1990). Despite the variety of different functions of HSP70, all the members of this heat shock family appear to play a role in protein synthesis by functioning as molecular chaperones (more in that section).

HSP 20 Family

In mammalian cells, only one ~25 to 30 kDa stress protein has been identified. It exists at very low levels under normal conditions but is highly induced by stress. Interestingly, these proteins share significant sequence homology with the α-crystallin proteins of the lens. As with a-crystallin, the low molecular weight (LMW) HSP appears to exist in very large configurations of 400,000 Da and after heat shock forms even larger structures of 2×10^6 Da (Arrigo et al., 1988). The LMW stress protein appears to be localized to the cytoplasm of the cell in close association with the Golgi complex but it relocates to the nucleus following a heat shock (Arrigo et al., 1988). There are at least 5 members of this HSP family in *Drosophila* and they appear to be differentially expressed during development (Cheney and Shearn, 1983; Pauli et al., 1989; Sirotkin and Davidson, 1982; Zimmerman et al., 1983). The developmental regulation of HSP28 may be

important for future research in this area in our models of puberty. In addition, some of the *Drosophila* LMW HSPs appear to be regulated by steroid hormones (Ireland and Burger, 1982). This is certainly evident in certain breast tumour cell lines where HSP28 is reported to increase following estrogen (Edwards et al., 1980). In addition, HSP28 seems to undergo rapid phosphorylation following treatment with phorbol esters or serum (Welch, 1985). A 27 kDa protein (HSP27) has also been implicated in estrogen action in human endometrial tissue, especially as an estrogen-regulated protein (Padwick et al., 1994).

HSP90 Family

HSP90 is one of the most abundant proteins in unstressed mammalian cells but its synthesis still increases after stress (Lai et al., 1984; Iwasaki et al., 1989; Hickey et al., 1989). In vertebrates, HSP90 exists in the cytosol and there appears to be another similar protein localized to the ER (Mazzarella and Green, 1987; Sorger and Pelham, 1987). In rats, HSP90-li can be localized to almost every neuron in the brain (Itoh et al., 1993). HSP90 mRNA also has a widespread distribution but labeling intensity varies between regions (Izumoto and Herbert, 1993). HSP 90 is encoded by two genes (a and b) and the protein appears to exist as a dimer with a large Stoke radius indicating a protein with a rodlike structure. Various biochemical studies have shown that HSP90 complexes with a number of cellular proteins including retroviral transforming proteins, cellular tyrosine kinases, steroid hormone receptors, actin and tubulin (Lindquist and Craig, 1988). Studies on the interaction of HSP90 with the steroid hormone receptor have shown that in the absence of steroid, HSP90 is complexed with the receptor and a variety of other proteins (Dalman et al., 1989; Howard et al., 1990). Following hormone binding, HSP90 dissociates allowing the steroid-receptor complex to bind to the DNA and initiate transcription. If HSP90 is artificially

removed from the steroid receptor complex, the receptor binds to the DNA in the absence of steroid (Sanchez et al., 1987). Dalman et al. (1991) summarized concisely that HSP90 sterically prevents the receptor from intercalating with the DNA by binding to the hormone binding site until the incoming steroid disrupts the receptor interaction with HSP90. In addition, initial binding of steroid receptor to HSP90 is needed to maintain the receptor in a hormone-responsive conformation (Bresnick et al., 1989). Interestingly, the thyroid hormone receptor and retinoid receptors do not seem to bind HSP90 (Dalman et al., 1990, 1991).

Heat Shock Proteins as Molecular Chaperones

The role of HSPs as molecular chaperones is to "stabilize non-native conformations of other proteins and to facilitate their correct folding by releasing them in a controlled manner" (Hendrick and Hartl, 1993; Hartl et al., 1994). This seems to occur during protein denaturation in adverse conditions and in *de novo* protein synthesis. Molecular chaperones such as HSP70 appear to identify partially denatured or unfolded proteins by the recognition of exposed hydrophobic surfaces rather than by a specific protein sequence (Gething and Sambrook, 1992; Hendrick and Hartl, 1993, Ellis et al., 1989). HSP70s do not bind to mature proteins but associate with unfolded polypeptide chains during their translation and translocation into subcellular organelles (Beckman et al., 1990; Nelson et al., 1992; DeShaies, 1988a,b; Chirico et al., 1988). The HSP70s appear to play a role in protein biogenesis during the early stages when the polypeptide chain is still extended. Other families of stress proteins have been implicated in protein synthesis and appear to act in a coordinated manner at different stages to promote protein folding and prevent aggregation.

The role of the 60 kDa family of stress proteins in the coordination of protein folding is best characterized by studies of homologous bacteria HSP,

GroEL (Becker and Craig, 1994). Members of this group of proteins are also known as "chaperonins" due to their pivotal role in protein synthesis (Ellis and van der Vies, 1991). It has been proposed that their distinctive structure of fourteen 60 kDa subunits stacked in two heptameric rings with a central cavity provides a separate compartment where protein folding can proceed (Hendrix, 1979; Hohn et al., 1979). Another protein, GroES or HSP10, is required for the complete function of HSP60. GroES is transcribed and forms a heptameric ring of 10 kDa which binds directly to the stacked ring structure inducing structural changes which are probably important in protein folding (Langer et al., 1992). Interestingly HSP70 and 60 appear to act sequentially in a molecular pathway which mediates the assembly and folding of proteins (Manning-Krieg et al., 1991). It appears that HSP70 first recognizes a hydrophobic sequence of an unfolded polypeptide and promotes the folding of the peptide into an intermediate state which then can be recognized by the HSP60 complex where folding is completed (Becker and Craig, 1994). The HSP60 family has been localized to the mitochondria of eukaryotic cells but recently there is some evidence for a 57 kDa protein called "tailless-complex polypeptide 1" (TCP-1) which may be a cytosolic member of the "chaperonins" (Gupta, 1990; Trent et al., 1991). Lewis et al., (1992) demonstrated that a homologous protein to TCP-1 derived from human cells is part of a double ring complex (950 kDa) similar in structure to the HSP60 chaperonins. The TCP-1 ring complex, known as TRiC, is comprised of 52 to 65 kDa subunits has been shown to prevent protein aggregation and to promote refolding in an ATP-dependent manner (Frydman et al., 1992). Lewis et al. (1992) have also suggested a cooperative role of HSP70 and TCP-1 based on their co-purification. The coordinated interaction of the various stress proteins in protein handling has been well illustrated in Figure 36 (Welch, 1993).

HSP and Neural Injury

The heat shock genes have attracted considerable attention from the neuroscience and medical research communities because they are expressed after a variety of neural insults (Brown, 1990) and are even expressed in humans in a number of disease conditions such as Alzheimer's (Perez et al., 1991). HSP72 can be induced in brain tissue by hyperthermia (Cosgrove and Brown, 1983; Nowak et al., 1990; Manzerra and Brown, 1990, 1992; David et al., 1994), ischemia (Dienel et al., 1986; Kawagoe et al., 1992a,b; Gonzales et al., 1989, 1991), seizures (Vass et al., 1989; Lowenstein et al., 1990; Sloviter and Lowenstein, 1992; Shimosaka et al., 1992) mechanical trauma (Currie and White, 1981) and excitatory amino acid analogues (Gonzales et al., 1989; Sharp et al., 1991a, 1992).

Figure 36. - Heat shock protein management of protein folding and cell distribution.

Several pathways for folding and distributing proteins inside cells are managed by stress proteins. In many cases different stress proteins seem to work in tandem. The cytoplasmic form of HSP70 binds to proteins being produced by the ribosomes to prevent their premature folding. The HSP70 may dissociate from the protein and allow it to fold itself into its functional shape (a) or to associate with other proteins and thereby form larger, multimeric complexes (b). In some cases, proteins are passed from HSP70 to another stress protein, TCP-1, before final folding and assembly occur (c). If the protein is destined for secretion, it may be carried to t! ꞓ endoplasmic reticulum and given to BiP or another related stress protein that directs its final folding (d). Other proteins are transferred to mitochondria or other organelles (e). Inside the mitochondrion, another specialized form of HSP70 sometimes assists the protein in its final folding (f), but in many cases the protein is passed on to a complex of hsp60 and hsp10 (g). The HSP60 molecule seems to serve as a "workbench" on which the mitochondrial protein folds. (Redrawn from Welch, 1993).
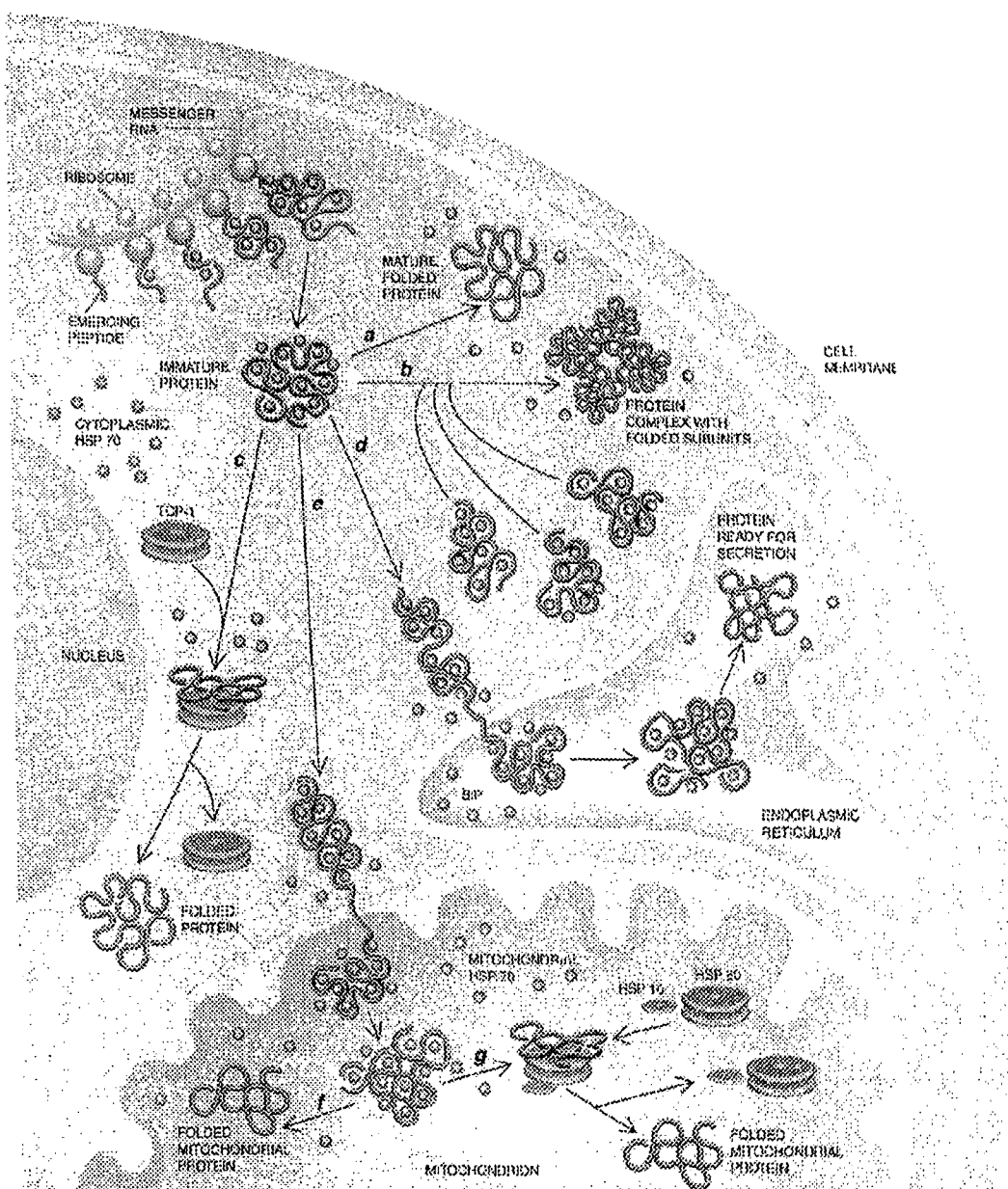
Fig. 36

Hypoxia/Ischemia

Hypoxia-ischemia is the best characterized of the neural stressors that induce HSP70 (reviewed by Sharp et al., 1993). Gonzalez et al. (1991) produced global ischemia for 5 -10 min. in adult rats using a combination of bilateral carotid artery (BCA) occlusion and hypotension. Eighteen to 24 hr. post-ischemia they observed variable HSP72-like immunoreactivity (-li) in the hippocampus, cortex, striatum, septum and thalamus of these rats. An incongruous HSP72 staining pattern was observed in the hippocampi following 5 vs. 10 min. of ischemia. Five min. occlusion of the carotid arteries induced HSP72 expression in columns of CA1α pyramidal neurons or throughout CA1 pyramidal and dentate hilar neurons. Conversely, 10 min. of ischemia induced staining in CA3 and dentate granule cells with minimal or no staining in CA1 and dentate hilar neurons. The authors hypothesize that the differential staining pattern is related to the relative vulnerability of the hippocampal cells to ischemia. The absence of CA1 staining following 1J min. of ischemia may be explained by the severity of the injury and the sensitivity of the CA1 pyramidal neurons causing widespread inhibition of protein synthesis including HSP72 expression. Contradictory results were reported by Tomioka et al. (1993) using a similar but longer (20 min.) model of global ischemia which is known to result mainly in the loss of CA1 neurons. This group discovered that HSP72-li was strongly expressed in neurons of both the CA1 and CA3 regions 1 day after ischemia. The induction of HSP72 was also quantified using Western blot analysis and the authors found that the level of inducible HSP70 in the injured CA1 region was the same as that in the surviving CA3 region. These results have also been reported by Nowak (1991) and Kawagoe et al. (1992a,b) in the gerbil and by Kumar et al. (1993) in the rat. They concurred with Tomioka et al. (1993) that HSP70 mRNA is expressed in both vulnerable (CA1) and resistant (CA3, dentate gyrus) regions of the hippocampus

in the post-ischemia period. Therefore, from these studies it is unclear whether the expression of HSP70 differentiates those neurons destined to die from those that will survive in the global model of ischemia.

HSP70 induction has also been reported in rat models of transient focal ischemia. Kawagoe et al. (1992a) used *in situ* hybridization to examine the distribution of HSP70 (HSP72) and HSC70 (HSP73) mRNA in forebrain at various time points after 30 min. occlusion of the middle cerebral artery (MCA). In this model of ischemia, the caudate is severely damaged whereas most of the cells in the cortex are fully preserved 7 days post-treatment (Nakano et al., 1990). In sham animals, levels of HSP70 mRNA were negligible but in experimental rats, expression was greatly increased 3 to 8 hr. after MCA occlusion. Overall, less HSP70 mRNA was present in the caudate compared to the cortex but peak mRNA levels occurred sooner in caudate (3 hr) than cortex (8 hr). In contrast to HSP70, HSC70 mRNA was present in sham animals in almost all areas of the brain. Ischemia increased the expression of the cognate gene in the same areas as the inducible gene. Levels of HSC70 mRNA were significantly reduced in the caudate 8 hr. post-ischemia. The authors hypothesize that the co-induction of HSP70 and HSC70 mRNA indicates a cooperative role for these proteins in the management of damaged and newly synthesized proteins in the cells of post-ischemic brain. Interestingly, less HSP mRNA was evident in the cells of the caudate relative to the cortex where cells are not damaged by focal ischemia.

Kinouchi et al. (1993) used *in situ* hybridization and immunocytochemistry to examine the heat shock response 4 and 24 hr. post-ischemia using permanent MCA occlusion. They localized HSP70 mRNA expression to both the area of MCA infarction and the outer perimeter of the lesion. HSP70 was only localized to the area outside the infarction. The appearance of HSP70 in the periphery of the lesion corresponds with the

"penumbra" or the area of ischemic tissue which may be rescued from infarction by pharmacological therapy (Kinouchi et al., 1993). The expression of HSPs after global or focal ischemia can be blocked by treatment with the NMDA antagonist, MK-801 (Graham et al., 1993) which implicates the neurotransmitter glutamate. This correlates with the evidence that shows a significant decrease in ischemic damage following MK-801 treatment (reviewed in Lipton and Rosenberg, 1994).

It is evident that there is a significant amount of circumstantial evidence that implicates the expression of HSP as a protective mechanism minimizing injury following a neural insult. This concept will be outlined in more depth in the Discussion.

Seizures

It is well known that sustained seizures result in pathological changes in neural tissue (Shimosaka et al., 1992). Several groups have investigated the induction of a neuronal stress response following seizures in animal models. Shimosaka et al. (1992) injected bicuculline into the area tempestas (AT) which is an epileptogenic site in the brain. In animals experiencing at least 12 min. of type IV EEG activity (as described in Lowenstein et al. 1990), HSP expression was noted in the ipsilateral deep piriform cortex and dorsal medial thalamus. With increased length of seizure activity, HSP70 was expressed bilaterally in the dorsal medial thalamus, amygdaloid complex and reuniens thalamic nucleus. Acid-fuschin staining which is indicative of neuronal damage (Auer et al., 1984) was observed in the same areas localized by HSP expression. Lowenstein et al. (1990) compared the distribution of HSP72-li with the predictable pattern of neuronal injury following flurothyl-induced status epilepticus. The degree of HSP70 staining was directly related to the duration of seizure activity and appeared to stain only neurons and ependymal cells. The authors noted that HSP72-li was

localized in neuronal regions which were both reversibly and irreversibly injured. Sloviter and Lowenstein (1992) used another well characterized model of excitation-induced neuronal damage to examine the expression of HSP. Stimulation of the main hippocampal afferent pathway for 24 hrs. evokes synchronous discharges of dentate granule cells but does not destroy them. However, vulnerable cell populations to this treatment include the dentate hilar mossy fibers, neuropeptide Y (NPY)-li neurons and CA3c pyramidal cells. One day after perforant pathway stimulation (2-6 hrs.), HSP72-li was noted in the susceptible dentate hilar and CA3c neurons but not in the dentate granule cells. Based on these results the authors believe that "HSP72 expression is a sensitive and specific indicator of excitation-induced and *potentially* injurious cell stress".

Excitatory amino acids

Gonzales et al. (1989) were the first group to investigate the expression of inducible HSP70 following systemic or intracerebral injection of the glutamate agonist and neurotoxin, KA (Coyle, 1981). No HSP72 immunostaining was present in rats treated with 6 mg/kg dose of KA at any time point. However, at the higher dose of 10 mg/kg, robust staining was observed in the hippocampus, neocortex, insular cortex, thalamic nuclei, cingulate cortex, septal nuclei, caudate putamen, bed nucleus of the stria terminals, anterior olfactory nuclei and olfactory bulb 24 to 48 hr. after KA treatment. Gonzales et al. (1989) appropriately described these neurons as having "Golgi-like stain" because there was dark, specific staining of cell bodies and neuronal processes. HSP72 was also observed in scattered glial cells. Following intracerebral injection of KA into dorsal hippocampus, a few HSP72-positive cells were found at the edge of the injection site and positive neurons were also found on distant structures such as the amygdala complex, mediodorsal thalamus and piriform cortex. Gonzales et

al. (1989) note that the pattern of HSP72-li agrees with the distribution of brain damage following KA injections and suggests that HSP72-li may be a useful marker of trauma or injury to the brain and a way of quantifying the severity of an injury.

Yee et al. (1993) examined HSP72 expression in relation to neuronal death at several time points after stereotaxic infusion of the glutamate agonist, NMDA, into the entorhinal cortex (EC). HSP72-li was maximal between 18 and 48 hr. following NMDA injection. A comparison of neuronal loss in the EC 7 days post-injection with maximum volume of HSP72-li and ubiquitin (Ub)-li indicates that a small population of cells expressing HSP72 and Ub survived the NMDA insult. The authors state that "HSP72 and Ub expression *in vivo* precedes and correlates with but does not necessarily lead to, neuronal death following glutamate receptor-mediated toxicity in the EC."

In contrast to what has been observed *in vivo*, glutamate treatment of cultured cerebellar granule cells did not induce a heat shock response (1 to 10 mM) (Lowenstein et al., 1991) even though NMDA has been shown to have a toxic effect on these cells (Cox et al., 1990). However, preinduction of heat shock protein by other means such as hyperthermia did protect cells from the toxicity of glutamate. The lack of a stress response following an insult is obviously a curious phenomenon and will be elaborated on in a later section (see Neural Injury and Absence of HSP Response).

Glutamate Antagonists and HSP

Non-competitive NMDA antagonists such as MK-801 have been implicated as possible therapeutic agents for stroke patients (Lipton and Rosenberg, 1994). It appears that a significant amount of ischemic damage can be prevented in experimental animals by blocking the effect of glutamate at the

NMDA receptor. Ironically, MK-801, phencyclidine (PCP) and ketamine also appear to produce reversible damage to posterior cingulate and retrosplenial cortex of rodent brains. For example, Sharp et al. (1991a) reported that i.p. injections of MK-801 (dose 0.1 to 5 mg/kg) or ketamine (40 to 100 mg/kg) induce HSP72 immunostaining in the same neurons that exhibited evidence of vacuolar damage. The peak level of HSP72-li occurred 24 hr. after MK-801 and lasted up to 7 days post-treatment. The greatest HSP72-li was observed with the 1 mg/kg dose and fewer neurons exhibited HSP72-li following the neurotoxic dose of 5 mg/kg of MK-801. It is possible that the necrotic and severely injured neurons did not produce HSP72. The time course and pattern of HSP72-li seen following ketamine treatment was identical to that seen with MK-801. It appears as though MK-801 and ketamine induced HSP72 expression primarily in injured neurons destined to survive. Sharp et al. (1992) have confirmed these results and demonstrated similar HSP72 expression following PCP. Pretreatment with the antipsychotic drugs, haloperidol and rimcazole, prevents the HSP response. These drugs appear to block dopamine and sigma receptors and one or both of these receptors may mediate the NMDA antagonist-induced injury.

Neural Injury and Absence of HSP Response

There are numerous studies which support the view that HSP expression is a marker of neural injury (Gonzales et al., 1989; for reviews see Brown, 1990; Sharp et al., 1993; Sharp and Sagar, 1994) but there are exceptions to this rule both *in vitro* and *in vivo* . For instance, Lowenstein et al. (1991) failed to identify a heat shock response in cultures of cerebellar granule cells following exposure to neurotoxic levels of glutamate (1 to 10 mM) but induction of HSP72 was observed in these cells following heat shock (42 to 43°C), sodium arsenite, and the calcium ionophore A23187. Saitoh et al. (1994) similarly found that heat

stress induced HSP72 expression in most cell lines derived from mouse, rat and human tissue but was absent in the mouse neuroblastoma cell line N18TG2. Kinouchi et al. (1993) reported the absence of HSP70-li overlying marked HSP70 mRNA expression within a neuronal lesion produced by permanent MCA occlusion. This phenomenon has also been observed by Nowak (1991) in the gerbil. Likewise, Lowenstein et al. (1990) reported that despite the close association between cell stress and HSP72 expression in their model of flurothyl-induced status epilepticus, some areas which are known to be necrotic following the experimental protocol, such as the substantia nigra, did not express HSP72-li. It has been suggested that the lack of HSP72 expression may represent a transcription or translation block brought about by a severe neuronal insult (Kinouchi et al., 1993). In brief, HSP72 expression appears to be a specific but not a sensitive test of cell stress in that the appearance of HSP72-li strongly suggests cell stress but the absence of HSP72-li does not rule out damage.

HSP and Neuronal protection

It appears that HSP expression is a relatively good indication that these cells have been stressed but it is not known whether the cells will survive or succumb to the stress. There is certainly evidence which suggests that HSPs protect cells from environmental insults. Pelham (1986) hypothesized that the expression of heat shock proteins may protect cells from injury by maintaining the tertiary structure of both normal and partially denatured proteins and by facilitating the transfer of large molecules across mitochondrial and ER membranes (Deshaies et al., 1988a, b). This hypothesis has been analyzed *in vivo* using the various models of ischemia (Simon et al., 1993; Liu et al., 1993; Nishi et al., 1993).

Simon et al. (1993) reported that a brief period of global ischemia sufficient to induce HSP72 but not cell death reduced the cortical infarct size produced by a subsequent (24 hr.) permanent MCA occlusion compared to "non-stressed" sham rats. Damage to the CA1 region of the hippocampus may be improved by "preconditioning" the brain with sublethal ischemia before a 2nd lethal length of ischemia (Kato et al., 1991; Kirino et al., 1991; Kitagawa et al., 1990; Liu et al., 1992; and Nishi et al., 1993). Interestingly, pretreatment with a heat shock also appears to protect hippocampal neurons from death from ischemic treatment (Chopp et al., 1989; Kitagawa et al., 1991). It seems plausible that the induction of HSP70 by any (i.e. hyperthermia, ischemia) initial non-lethal treatment plays a role in "preconditioning ischemic tolerance." Kato et al. (1992) have shown that ischemic tolerance can be inhibited by administering MK-801 during the first sub-lethal ischemic event.

The length of time between the initial stressful stimulus and the lethal insult appears to be important in the generation of the protective state. Kato et al. (1991) have reported that the interval between non-lethal (3 min.) and lethal (6 min,) ischemia must be between 1 day and 7 days for the generation of a damage-resistant state. Liu et al. (1993) noted that HSP70 synthesis occurs 1 to 7 days after sublethal ischemia, closely paralleling the occurrence of ischemic tolerance after pre-conditioning. Most of the evidence implicating HSP in the generation of ischemic tolerance, however, are correlation studies and do not prove that HSPs prevent cell damage. Transfection of proteins into neuronal cells and demonstrating thermal or ischemic tolerance or blocking the action of HSP with antibody would be a more direct line of experimentation. For instance, interruption of HSP72 expression after a mild heat shock blocks the development of thermotolerance in non-neuronal cells *in vitro* (Johnston and Kucey, 1988; Riabowl et al., 1988). Barbe et al. (1988) demonstrated that a brief exposure to

above ambient temperatures increased the synthesis of HSP70 mRNA and HSP70 in the retina and seemed to prevent the loss of photoreceptor cells (PRC) after 24 hr. exposure to a bright light (250 foot-candles). Intravitreal injections of purified HSP 70 increased PRC survival following exposure to damaging levels of light.

As observed in the models of ischemia, the heat shock response also protects cultured neurons from glutamate toxicity (Rordorf et al., 1991; Lowenstein et al., 1991). Heat treatment (42.2°C for 20 min.) of cultured cortical glia and neurons markedly induces HSP70 mRNA, 90 min post-treatment and increases synthesis of 72 and 85 kDa HSPs (Rordorf et al., 1991). Cell survival was measured by the number of non-pyknotic neurons and the amount of lactate dehydrogenase (LDH) leakage into the medium before and after glutamate exposure. Based on these parameters, it appears that either 4 or 24 hr. after heat shock, the heat shock treatment decreased the excitotoxic damage caused by glutamate. The protective effect was not evident 48 hr. after heat shock. In addition, the protective effects of hyperthermia were blocked by exposure of the cultured cells to inhibitors of RNA or protein synthesis (actinomycin D and cycloheximide, respectively) between heating and exposure to glutamate. A similar set of experiments was conducted by Lowenstein et al. (1991) using cultures of cerebellar granule cells. They also concluded that prior induction of heat shock proteins was associated with protection from glutamate toxicity (glutamate range: 100 μM to 5 mM). Rordorf et al. (1991) suggests that HSP protection of neuronal cells from excitotoxic processes may minimize brain injury in specific pathologic conditions.

Conclusion

A wide array of neuronal insults including the glutamate agonists and antagonists simultaneously cause cellular damage and provoke the transcription of stress proteins. As such, the immunocytochemical localization of HSP appears to be a convenient tool for examining cell stress in the context of our models of puberty and will be the focus of the research in this chapter. There is sufficient evidence to hypothesize that in our neonatal and juvenile rats treatment with EAA may cause neuronal damage. To test this hypothesis we will examine the expression of heat shock proteins in the same brain regions which have been shown to transiently express Fos following treatment with NMDA and MSG.

Material and Methods

I. Animals

Female Sprague Dawley rats housed under the same conditions described in Chapter 2 were used in this experiment.

II. Drugs

See the Material and Methods section of Chapters 2 and 3.

III. Immunocytochemistry for HSP72-li

The procedure used in the detection of HSP72-li is almost identical to that described for FLI in Chapter 3 (Material and Methods section). The following changes to our standard protocol were used for HSP72-li ...

(i) Tissue sections were incubated in 3% normal horse serum (NHS; Gibco Laboratories, NY; 200 6050AG) and 0.1% bovine serum albumin (BSA; Sigma, MO; A-4503) in PBS and TX for 1 hr. to block non-specific binding by the primary (1°) antibody.

(ii) After the blocking step, the sections were incubated for 5 days at 4°C with a mouse monoclonal HSP72 antibody (StressGen Biotechnologies Corp., Victoria, B.C.; SPA-810) at a dilution of 1:2000 in 3% NHS.

(iii) After the incubation, the sections were washed three times (10 min. per wash) in PBS and TX and incubated in rat-adsorbed biotinylated horse anti-mouse antibody (Vector Laboratories; BA 2001) at a dilution of 1:500 in 3% NHS and 1% TX at room temperature for 1 hr.

(iv) After three washes, the sections were incubated with avidin-biotin-peroxidase complex (Elite ABC kits; Vector Laboratories; PK-6100) at a dilution of 1:200 (5 ml each of solution A and B/ml) in PBS at room temperature for 1 hr.

IV. Experiments

(1) The Effect of NMDA Treatment on HSP72-li in the CNS

Female rats were injected with NMDA (20 mg/kg; s.c.; n=6) or 0.9% saline (0.1 ml; s.c.; n=4) at 2100 h on P28. At 0900 h on P29 (12 hrs. post-injection), half the rats from each group were deeply anaesthetized, perfused and their brains removed for sectioning. This was repeated at 2100 h on P29 (24 hr. post-injection) with the remaining rats. Brain nuclei which are known to increase *c-fos* expression after NMDA treatment (ARCN), PVN, AMPOA at OVLT, SFO, AP and SON were sectioned at 50 μm for HSP72 immunocytochemistry. The following areas of the brain were located in the 50 μm coronal sections and alternate sections were processed for HSP72-li: AMPOA (Bregma -0.26 to -0.40), SFO (Bregma -0.80 to -1.40), PVN (Bregma -1.80 to -2.30), SON (Bregma -0.80 to -1.80), ARCN (Bregma -2.12 to -3.30) and AP (Bregma -13.68 to -14.08) (Paxinos and Watson, 1986). Sections from the ARCN were arranged in series to examine the pattern of HSP72-li in the nucleus from rostral to caudal.

(2) The Time Course of NMDA Induced HSP72-li in the MBH

Female rats were injected with NMDA (20 mg/kg; s.c.) or 0.9% saline vehicle (0.1 ml; s.c.) daily at 1300 h from P24 to P27. At age 28 days both saline- and NMDA-treated groups were injected with 20 mg/kg of NMDA in 0.1 ml saline (s.c.) at 1300 h. At various time points (4, 8, 12, 24, 48, 72 h, 7 days, 14 days) after the NMDA injection on day 28, rats from each group (NMDA (x1) vs. NMDA (x5); n=4/group) were deeply anaesthetized, perfused and the brains removed for sectioning. Six coronal sections (50 μm) from the caudal portion of the MBH of each animal (Bregma -2.80 to -3.30 mm) were processed for HSP72-li.

(3) HSP 72-li in the ARCN of Female Rats after Increasing Doses of NMDA

At 2100 h on P28, female rats were injected with 0.9% saline vehicle (0.1 ml; s.c.; n=3) or various doses of NMDA (5, 10, 20 and 40 mg/kg; s.c.; n=4/dose). Twelve hrs. after the injection (0900 h on P29), the rats were anaesthetized, perfused and the brains were prepared for immunocytochemistry as described previously.

(4) The Ontogeny of NMDA-Induced HSP 72-li in the ARCN of Female Rats

Several litters of female rat pups were housed in the IWK AQ and examined for the response of the *HSP72* gene to NMDA at various ages between P2 and P28. Female rats were also examined at P60 for HSP72 expression following NMDA treatment. On P2, female pups were injected with 0.9% saline (0.1 ml; s.c.; n=8), NMDA (3 mg/kg; s.c.; n=12) or MSG (2 or 4 mg/g; s.c.; n=12/dose). At 4, 12, 18 or 24 hr. post-injection (n=2-3/time pt.) the pups were anaesthetized, perfused and brains removed for sectioning (100 μm). In the P9 group, female pups were injected with saline (0.1 ml; s.c.) or NMDA (5 mg/kg; s.c.) and perfused 12 or 24 hr. after injection (n=3/group). All of the coronal brain sections through the ARCN of P2 and P9 pups were processed for HSP 72-li. The juvenile female rats (P15, P21, and P28) were injected with saline (0.1 ml; s.c.) or NMDA (20 mg/kg; s.c.) and sacrificed 12 or 24 hr. after injection (n=3-4/group). The young adult female rats (P60) were injected with saline (0.1 ml; s.c.) or one of two doses of NMDA (20 or 60 mg/kg; s.c.) and perfused 12 and 24 hr. post-injection. The brains from the P15, P21, P28 and P60 groups were sectioned (50 μm) and 6 sections from each animal were processed for HSP72-li as described previously.

Note that different doses of NMDA were used with the various age groups due to the decrease in sensitivity to NMDA with age. For instance,

relatively low doses of NMDA were used in the neonatal rats (3 and 5 mg/kg) because 20 mg/kg of NMDA is lethal (see MacDonald and Wilkinson, 1992).

(5) The Effect of Estradiol Benzoate on HSP72-li in the MBH and AMPOA

We examined the effects of estradiol benzoate (EB), on HSP 72-li in the hypothalamus and POA. Juvenile female rats (P28) were administered EB (10 µg; in sesame oil; s.c.) or oil vehicle (0.1 ml; s.c.) at 2100 h. Twelve (n=3/group; 0900 h on P29) or 24 hrs. (n=3/group; 2100 h on P29) after treatment, the rats were deeply anaesthetized, perfused and brains removed for sectioning. Tissue sections (50 µm) from the AMPOA and MBH were chosen as described in experiment 1 and 2, respectively, and processed for HSP72-li as described previously.

(6) MK-801 and APV Block of NMDA Induced HSP72-li in the ARCN

We examined whether the non-competitive NMDA antagonist, MK-801, or the competitive antagonist, APV, could block NMDA-induced HSP72-li in the MBH of juvenile female rats. At 28 days of age, four groups of rats were injected with either saline vehicle (0.1 ml; s.c.), MK-801 (0.1 or 1.0 mg/kg; s.c.) or APV (20 mg/kg; s.c). Half of each treatment group was administered saline (0.1 ml; s.c.) or NMDA (20 mg/kg; s.c.) 30 min. after the first injection. Another group of rats was administered MK-801 (0.1 mg/kg; s.c.) 15 min. prior to NMDA (20 mg/kg; s.c.). The 9 groups of animals were anaesthetized and perfused 12 hrs. and 24 hrs. (n=2- 5/group/time pt.) after the last injection for HSP72-li. Coronal sections (50 µm) through the MBH were chosen as in experiment 2 and processed for HSP72-li as described previously.

(7) The Effect of Multiple MK-801 Injections on HSP72-li in the ARCN

In this experiment we examined the effects of multiple MK-801 injections on HSP72-li using a protocol which is known to significantly delay but not prevent the onset of puberty in female rats (MacDonald & Wilkinson, 1990). Experimental animals were injected daily with saline vehicle (0.1 ml; s.c.) or MK-801 (0.1 mg/kg; s.c.) from the age of 27 to 32 days at 1300 h. A few rats that had been treated with saline from P27 to P31 were given MK-801 (0.1 mg/kg; s.c.) on P32 at the same time. Twelve or 24 hrs. after the last injection on P32, the rats (n=3/group) were deeply anaesthetized, perfused and brains removed for sectioning (50 µm) as in experiment 2. Sections from the retrosplenial cortex and cingulate cortex were included as Sharp et al. (1991a) have observed HSP72 expression in these areas following MK-801 treatment.

(8) The Effect of Neonatal MSG on NMDA-Induced HSP 72-li in the ARCN

Neonatal female pups were treated with saline vehicle (0.1 ml; s.c.) on P2 or MSG (2 mg/g; s.c.) on P2 or with MSG (4 mg/g; s.c.) on P2, P4, P6 and P8. At 28 days of age rats were injected with either saline (0.1 ml; s.c.) or NMDA (20 mg/kg; s.c.) at 0900 h (n=3/group). Twelve hrs. post-injection (2100 h on P28) the rats were anaesthetized, perfused and brains removed for sectioning (50 µm).

(9) The Effect of Other Excitatory Amino Acids on HSP72-li in the CNS

We examined the pattern of HSP72-li in the CNS of young female rats treated with KA at doses which are known to cause neuronal damage (Gonzalez et al., 1989; Schmued et al., 1993). Female rats (P28) were injected with 0.9% saline (0.1 ml; s.c.) or KA (8 or 10 mg/kg; s.c.) at 2100 h. Twelve or 18 hrs. after the injection, rats from each group (n=3-6/group) were anaesthetized, perfused and brains removed for sectioning. The caudal portion of the MBH was sectioned as described in experiment 2 and processed for HSP 72-li.

V. Photography

Photographs were taken using an automatic photomicrographic system (Leica, Switzerland; WILD MPS 46/52) attached to a Leitz (Wetzlar, Germany) Laborlux S microscope. The film used was Ilford Pan F Plus (ASA 50). The majority of photographs were taken with the 10x objective. The films were developed and photographs were printed in Medical Computing and Media Services, Dalhousie University.

Results

(1) The Effect of NMDA Treatment on HSP72-li in the CNS

Figure 37 and 38 illustrate the effects of NMDA treatment on HSP72-li in various areas of the CNS of the peripubertal (P28) female rat. The most abundant immunostaining was found in the ARCN 12 hr. after treatment (arrowheads indicate HSP72-positive cells in Fig. 37A, B, 38A). Staining was still intense at the 24 hr. time point but the number of HSP72-positive cells had declined (not shown). The nucleus, cell body and occasionally, neuronal processes (see arrows) of the HSP72-positive cells are stained (Fig. 37C, D - magnified from position of star in Fig. 37A, B). Several faint HSP72-li cells were evident in the subfornical organ (SFO; see arrowhead Fig. 38B) and the AP at 12 hrs and the intensity of the stain increased slightly by 24 hr. post-treatment (see arrowheads Fig. 38C and F, respectively). At the level of the AP, several HSP72-positive cells were also localized in the nucleus of the solitary tract (see arrow Fig. 38C). The rare HSP72-positive cell was detected in the AMPOA (see arrowhead Fig. 38E) at the 24 hr. time point only. No specific staining was noted in the supraoptic nucleus, suprachiasmatic nucleus or paraventricular nucleus of the hypothalamus at any time point (not shown). No HSP72 immunostaining was evident in the saline-treated controls (Fig. 38D) or in the sections incubated in horse serum in the absence of HSP72 antibody (not shown).

Figure 39 summarizes the effects of NMDA on HSP72-li 12 hr. after treatment in the rostral (Fig. 39A) to the caudal (Fig. 39E) portions of the ARCN/ME. Note that the pattern of HSP72-li follows the boundaries of the arcuate nucleus fairly closely in all tissue sections. As such, the majority of immunostaining is observed in the more medial-caudal portions of the hypothalamus.

Figure 37. - Photomicrograph of HSP72-li in the ARCN of juvenile female rats treated with NMDA.

HSP72-li in two coronal sections (A, B) through the ARCN of a P28 female rat treated 12 hrs. previously with NMDA (20 mg/kg; s.c.). Portions of the photomicrographs were magnified (see star) and are shown in C and D. Note that the nucleus, cell body and neuronal processes (see arrows) exhibit HSP72-li following NMDA treatment. Scale bar = 100 μm.
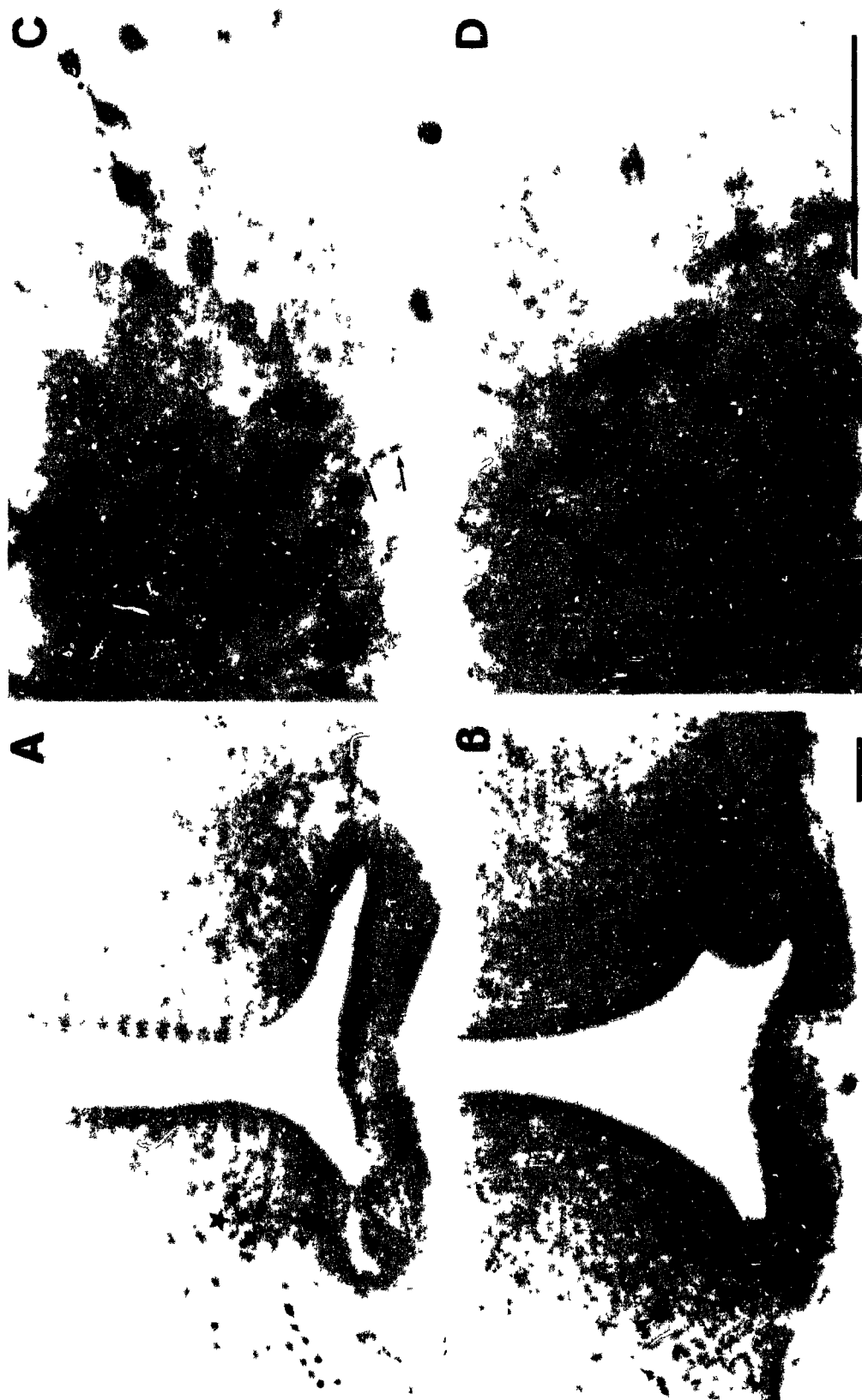
Fig. 37

Figure 38. - Photomicrographs of HSP72-li in the ARCN, SFO, AP and AMPOA following NMDA.

HSP72-li is observed in the ARCN (A), SFO (B), AP (C,F) and AMPOA (E) of P28 female rats 12 hrs. post-NMDA (20 mg/kg; s.c.) treatment (n=4-6/group). The most intense immunostaining is observed in the ARCN with minimal staining in the SFO and AP. The rare cell in the AMPOA displayed HSP72-li following NMDA (see arrow). HSP72-li was not observed in saline-treated controls (D) or tissue sections from NMDA-treated rats incubated without 1° antibody (not shown). Scale bar = 100 μm.
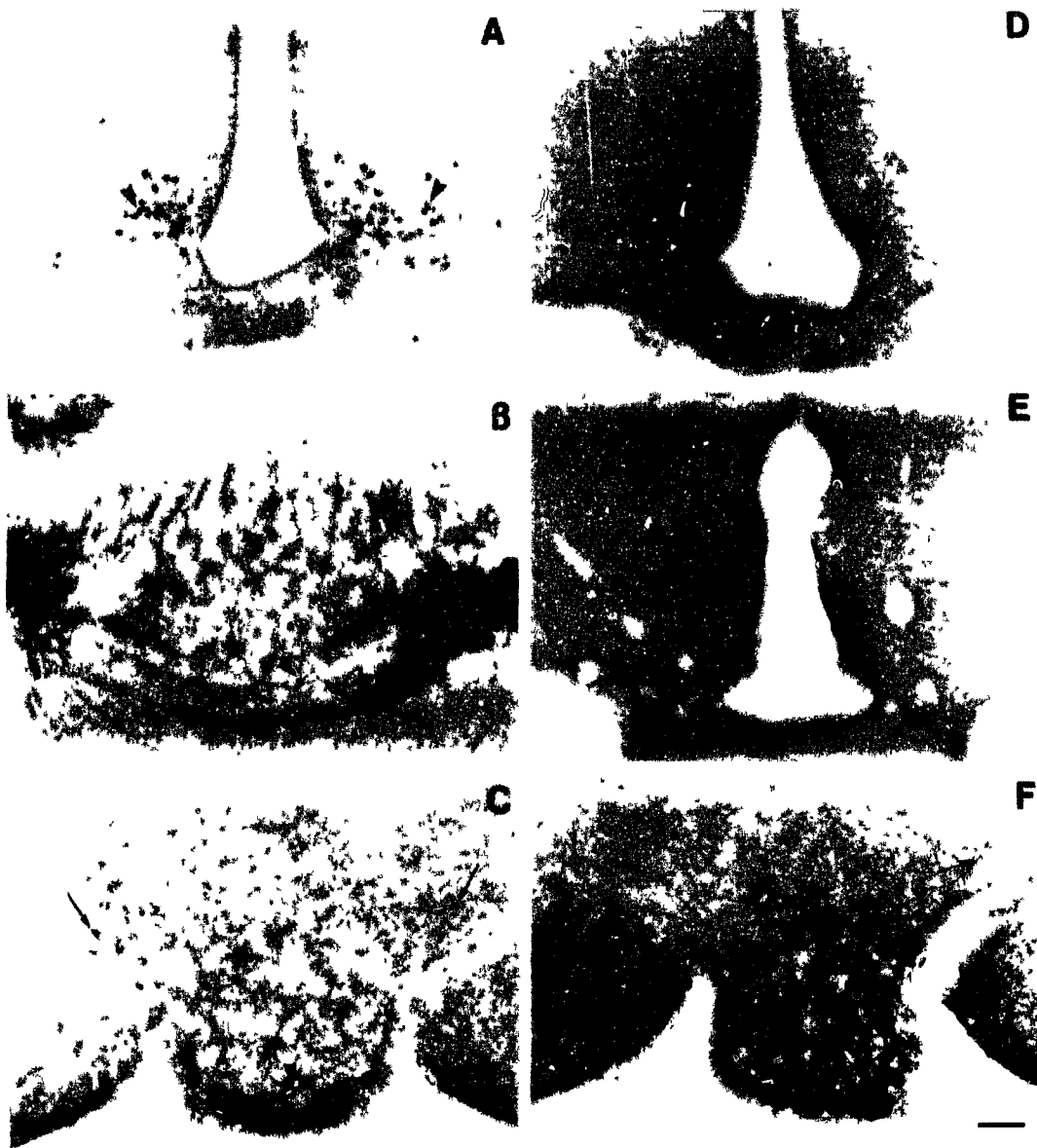
Fig. 38

Figure 39. - HSP72-li in serial sections of ARCN of NMDA-treated rat.

Photomicrographs of HSP72-li in serial sections through the ARCN of P28 female

rats 12 hrs. after treatment with NMDA (20 mg/kg; s.c.). HSP72-positive cells

follow the boundaries of the ARCN from the most rostral (A) to caudal (E)

sections corresponding to Bregma -2.12 mm to -3.30 mm. (Scale bar = 100 μm).
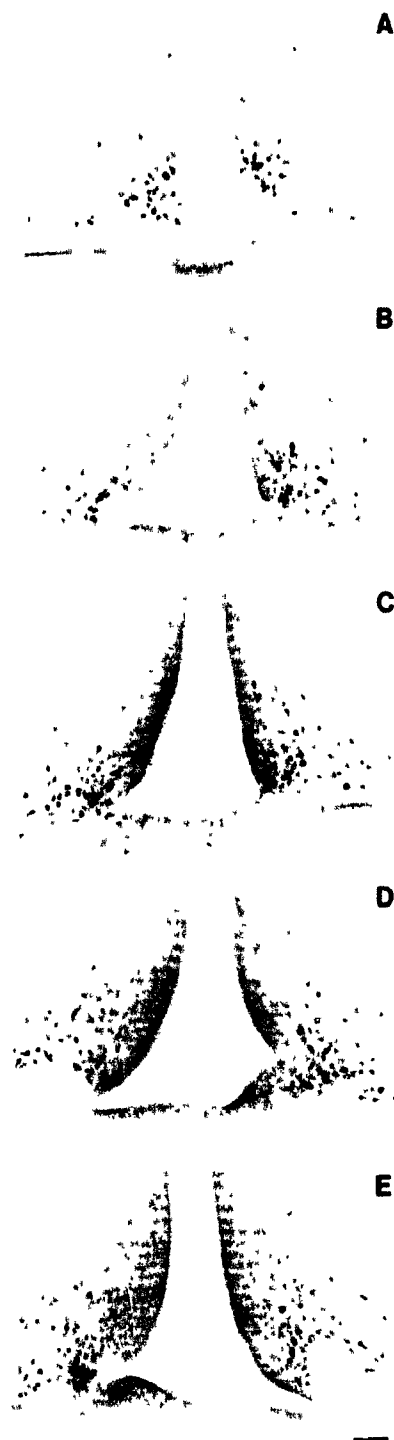
Fig. 39

(2) The Time Course of NMDA Induced HSP72-li in the MBH

We examined the time course of NMDA-induced HSP72-li in the ARCN/ME area of P28 female rats receiving NMDA for the first time or the fifth time (NMDA (x1) vs. NMDA (x5); as per our NMDA model of puberty; Fig. 40). The time course of NMDA-induced HSP72-li is very similar in both the NMDA (x1) and NMDA (x5) group. Peak immunostaining for HSP72 is observed 8 hr. post-injection (Fig. 40B and 40G). The number of HSP72-li cells declines by 24 hr. post-injection but the cells which remain immunopositive are quite densely stained (see arrowheads Fig. 40C and 40H). NMDA-induced HSP72-li appears to decline gradually, with fewer HSP72-positive cells evident at 48h (not shown) and 72h (Fig. 40D and 40I) post-injection. In general, HSP72-li in both NMDA (1) and NMDA (5) groups, disappears 7 days after treatment (Fig. 40E, J) but in some animals an occasional HSP72-positive cell still remains (see arrow Fig. 40E). No HSP72-positive cells in either group were found 14 days post-treatment (not shown).

The major difference in HSP72-li in the ARCN/ME between the NMDA (x1) and NMDA (x5) group is the number of HSP-positive cells and the intensity of immunostaining evident 4, 8 and 24 hrs. post-injection At the 4 hr. point in the NMDA (x1) group, HSP72 immunostaining is very faint (see arrows Fig. 40A). In contrast to this, the NMDA (x5) group appears to have more immunoreactive cells and more densely stained cells (see arrows Fig. 40F). Similarly, at the 8 hr. time point the number of HSP72-positive cells is much greater in the rats receiving the drug for the fifth time (Fig. 40G) as opposed to those receiving treatment for the first time (Fig. 40B). The difference between groups becomes less evident as the time post-injection increases. For example, in the two treatment groups at the 48 (not shown) and 72 hr. (see arrowheads Fig. 40D and 40I) time point, the number of HSP72 immunopositive cells are quite comparable.

Figure 40. - The time course of NMDA-induced HSP72-li in the ARCN of juvenile female rats.

The NMDA (x1) group was treated with NMDA (20 mg/kg; s.c.) for the first time on P28 and examined for HSP72-li 4, 8, 24, 72 hrs., 7 and 14 days after injection (A to E, resp.). The NMDA (x5) group was treated with NMDA (20 mg/kg; s.c.) for the fifth consecutive day (P24 to P28) at 28 days of age and examined for HSP72-li at the same time points as the NMDA (x1) group (F to J, resp.). Scale Bar = 100 μm. Saline-treated rats (not shown) had no HSP72-positive cells in the ARCN.

A · 4 hr.
B · 8 hr.
C · 24 hr.
D · 72 hr.
E · 7 days
F · 4 hr.
G · 8 hr.
H · 24 hr.
I · 72 hr.
J · 7 days

NMDA (x1)   NMDA (x5)
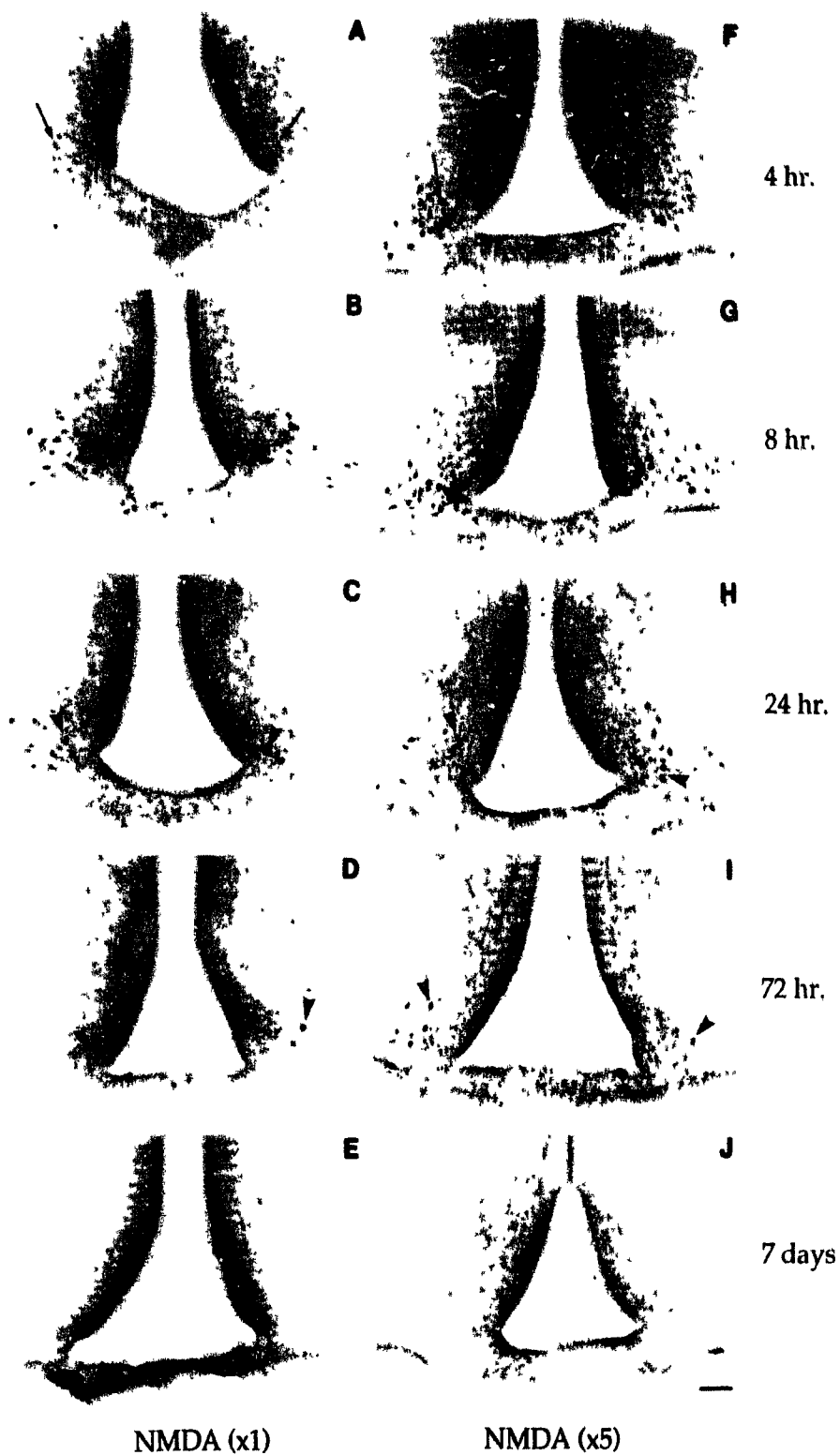
Fig. 40

(3) HSP72-li in the ARCN of Female Rats after Increasing Doses of NMDA

NMDA treatment causes a dose-related increase in HSP72-li in the ARCN/ME of prepubertal female rats. A few HSP72-li cells are evident 12 hr. after treatment with 5 mg/kg of NMDA (see arrowheads Fig. 41A). The number of HSP72-positive cells increases gradually following 10, 20 or 40 mg/kg of NMDA (Fig. 41B, C and D, respectively). It appears that with increasing doses of NMDA, the pattern of HSP expression enlarges along a gradient away from the ventricle. A higher dose than 40 mg/kg of NMDA was not used due to the adverse behavioural effects of the drug. Saline-treated control rats did not express HSP72-li in the ARCN/ME (Fig. 41E).

(4) The Ontogeny of NMDA-induced HSP72-li in the ARCN of Female Rats

Figure 42 illustrates the effects NMDA (or MSG) on HSP72-li in the ARCN/ME area of female rats at various ages from the neonate (P2) to the prepubertal stage (P28) at either 12 or 24 hr post-injection. In the P2 females, at 12 and 24 hrs. after treatment, neither MSG (2 or 4 mg/g; not shown) nor NMDA (3 mg/kg; Fig. 42A and F) induced HSP72-li in the ARCN/ME. Similarly, NMDA treatment (5 mg/kg; 42B and G) of P9 female pups did not trigger an increase in HSP72 expression in the ARCN/ME at any time point. However, the P15 females exhibited a pronounced HSP72 response to NMDA, evident at both the 12 and 24 hr. time points (Fig. 42C and H). Similarly, the P21 (Fig. 42D and I) and P28 (Fig. 42E and J) female rats responded to NMDA treatment with the induction of HSP72-li in the ARCN/ME area. In all groups that responded to NMDA with an increase in HSP72-li, staining is greatest at the 12 hr. time point. The P21 group appears to have the greatest HSP72 response to NMDA treatment. The area exhibiting HSP72-li becomes smaller and more restricted to the tissue closest to the ventricle with increasing age (see arrows and compare 42C with E).

Figure 41. - A HSP72-li dose-response in the ARCN of P28 female rats after NMDA.

Photomicrographs of a dose response for HSP72-li in the ARCN of juvenile (P28) female rats 12 hrs. after treatment (s.c.) with increasing doses of NMDA (A: 5 mg/kg; B: 10 mg/kg; C: 20 mg/kg; D: 40 mg/kg) or saline (E: 0.1 ml; s.c.). Scale bar = 100 μm.

5
mg/kg

10
mg/kg

20
mg/kg

40
mg/kg

Saline
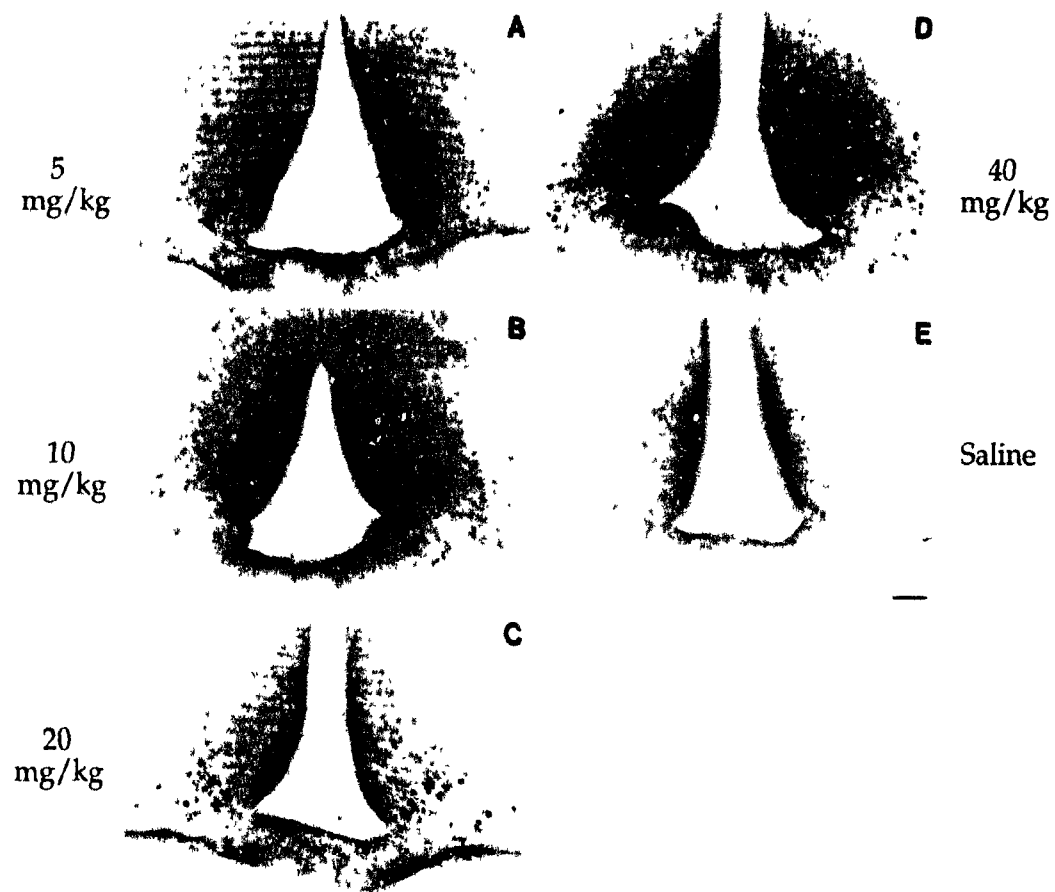
Fig. 41

Figure 42. - The ontogeny of glutamate-induced HSP72-li in the ARCN of juvenile female rats.

Photomicrographs showing the ontogeny of the HSP72 response to glutamate agonists in female rat brain. The rats were given separate doses of NMDA depending on their age (P2: 3 mg/kg; P10: 5 mg/kg; P15 to P28: 20 mg/kg) and examined for HSP72-li in the ARCN 12 and 24 hrs. after treatment (P2: A and F; P10: B and G; P15: C and H; P21: D and I; P28: E and J). Neonatal P2 rats were also administered MSG (2 or 4 mg/g) and examined 12 and 24 hrs. post-injection (not shown). HPS72-li is not apparent until P15 and is maximal at P21. Scale bar = 100 μm.

A

F

P2

B

G

P10

C

H

P15

D

I

P21

E

J

P28

12 hr.

24 hr.
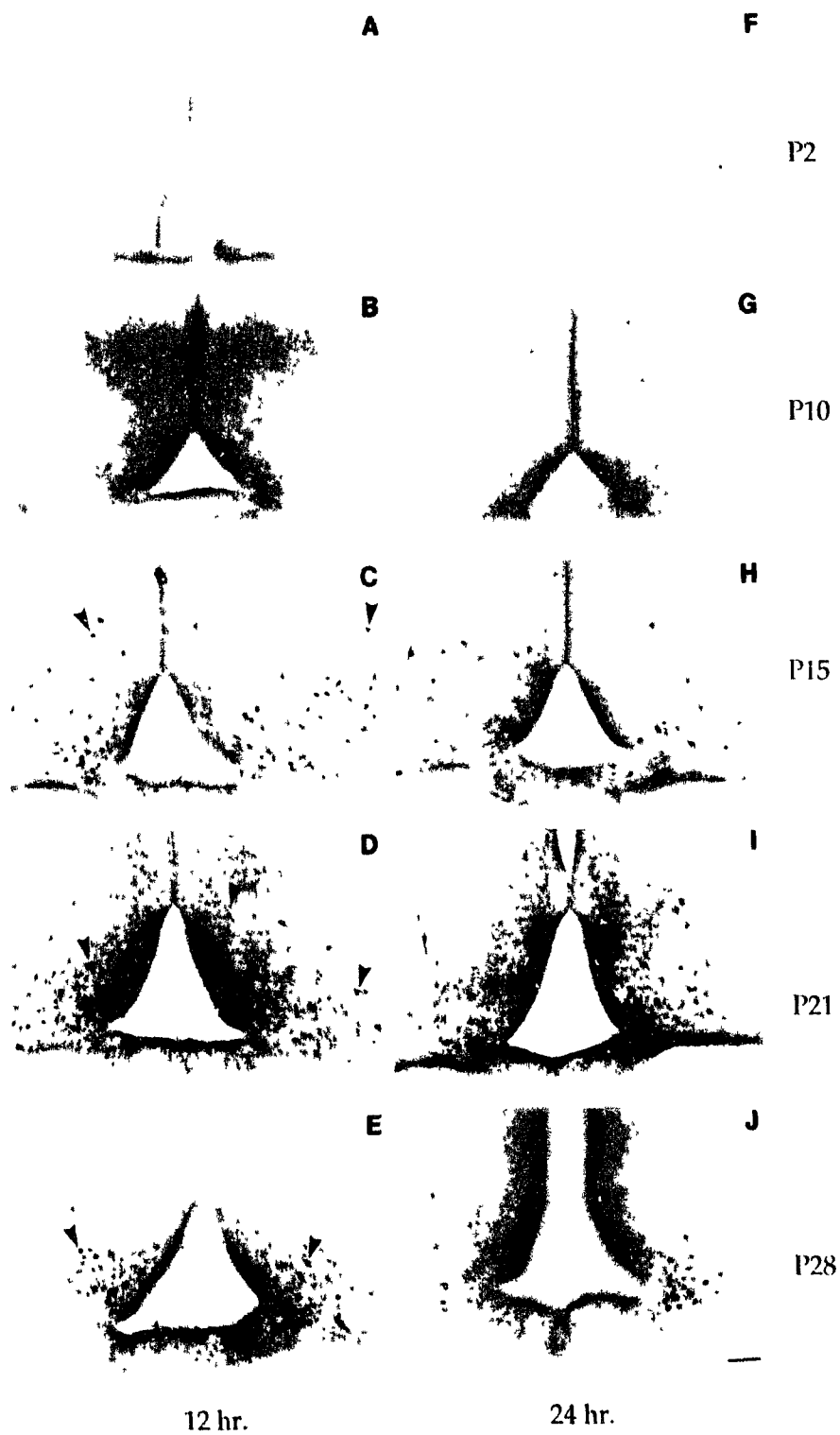
Fig. 42

Young adult female rats (P60) were administered saline or one of two doses of NMDA (20 or 40 mg/kg) and HSP72-li in the ARCN/ME was examined 12 hrs. post-treatment. Figure 43 illustrates that a heat shock response to NMDA is present in the older animals (Fig. 43A and B), but is significantly attenuated compared to that observed in the prepubertal female rats. Control rats did not exhibit any HSP72-li in the ARCN/ME (Fig. 43C).

(5) The Effect of Estradiol Benzoate on HSP72-li in the MBH and AMPOA

EB did not stimulate HSP72-li in the ARCN or AMPOA of juvenile female rats. Staining was absent at 12 and 24 hrs. in both control and steroid-treated groups (not shown).

(6) MK-801 and AP-5 Block of NMDA-Induced HSP72-li in the ARCN

We examined the effects of a noncompetitive channel blocker, MK-801, on the ability of NMDA to induce HSP72-li in the ARCN/ME of P28 female rats. Saline pre-treatment did not block NMDA-induced HSP72-li (arrowheads indicate HSP72-positive cells; Fig. 44A). Neither dose of MK-801 (0.1 or 1.0 mg/kg) triggered HSP72 expression in the ARCN/ME 12 hr. post-injection (Fig. 44E and F). However, treatment with MK-801 30 min. prior to an injection of NMDA either partially (0.1 mg/kg dose; see arrowheads Fig. 44B) or completely (1.0 mg/kg dose; Fig. 44C) abolished NMDA-induced HSP72-li in the ARCN/ME area. Interestingly, MK-801 (0.1 mg/kg) administered 15 min. prior to NMDA did not suppress HSP72-li (not shown).

We also examined the ability of a competitive antagonist, APV, to block the effects of NMDA on HSP72-li in the ARCN/ME of P28 female rats. As seen previously, APV (20 mg/kg) alone did not trigger HSP72-li in the hypothalamus (Fig. 44G) but, pre-treatment with APV (30 min.) completely suppressed the effects of NMDA-induced HSP72-li (Fig. 44D).

Figure 43. - The effect of different doses of NMDA on HSP72-li in the ARCN of adult female rats.

Photomicrographs of HSP72-li in the ARCN of adult female rats (P60) 12 hrs. after NMDA (s.c.) (A: 20 mg/kg; B: 40 mg/kg) or saline vehicle (0.1 ml; s.c.) (C). Scale bar = 100 μm.

A — NMDA 20 mg/kg

B — NMDA 40 mg/kg

C — Saline
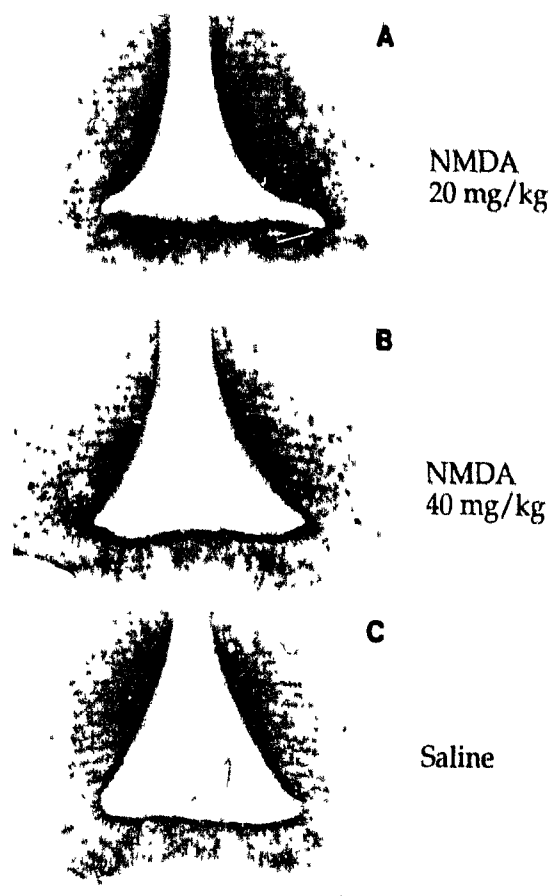
Fig. 43

Figure 44. - Blockade of NMDA-induced HSP72-li in the ARCN of juvenile female rats with MK-801 or APV.

Photomicrographs of HSP72-li in the ARCN of peripubertal female rats (P28) 12 hrs. after injection (s.c.). HSP 72-li following treatment with NMDA (20 mg/kg; s.c.) which was preceded by 30 min. by an injection with saline (0.1 ml; s.c.), MK-801 (0.1 or 1.0 mg/kg) or APV (20 mg/kg) is shown in A to D, respectively. The effect of the antagonists alone on HSP72-li are shown in E (MK-801, 0.1 mg/kg), F (MK-801, 1.0 mg/kg) and G (APV, 20 mg/kg). Scale bar = 100 μm.

NMDA

MK-801 0.1
+ NMDA

MK-801 1.0
+ NMDA

APV
+ NMDA

MK-801
0.1

MK-801
1.0

APV
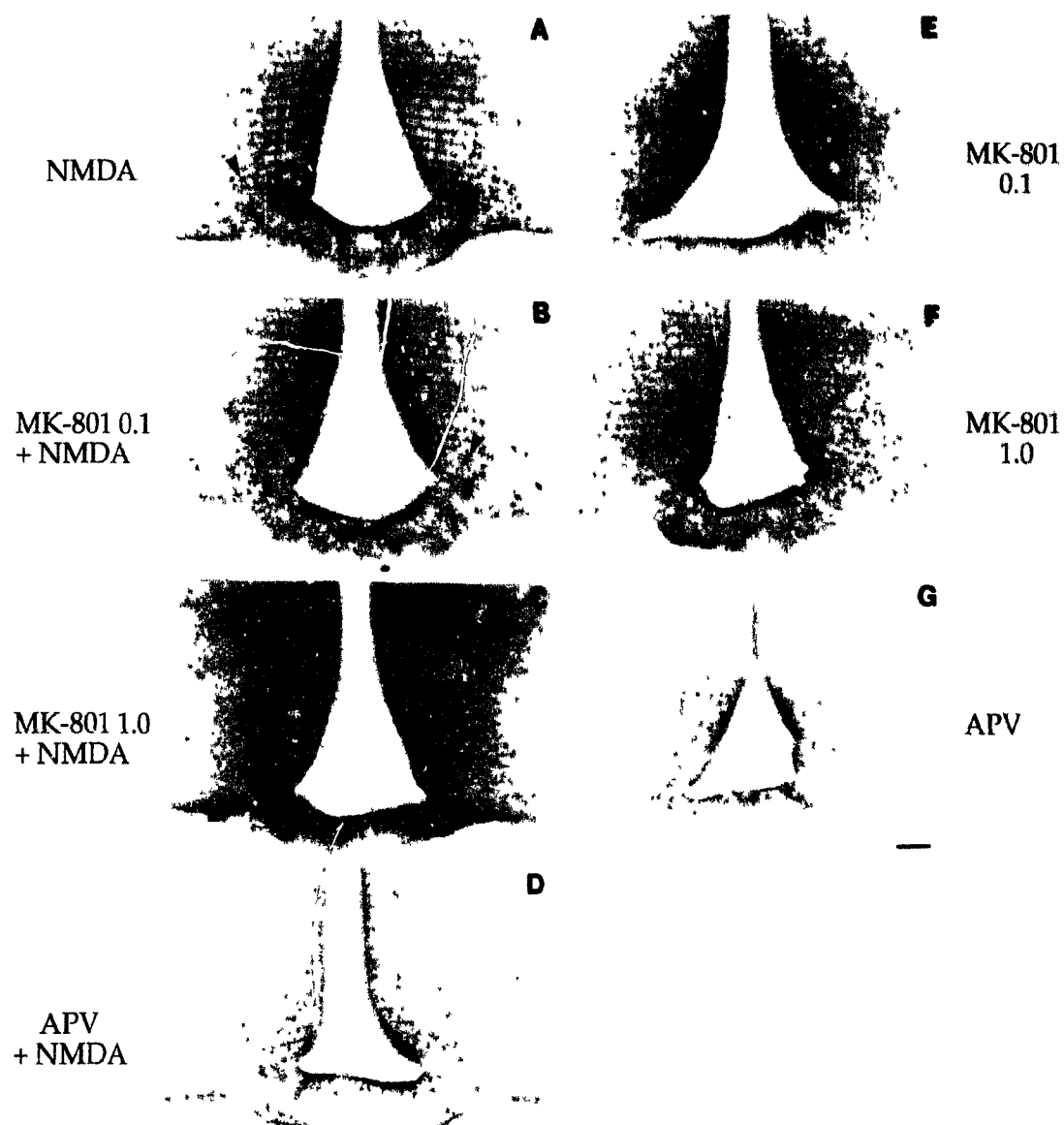
Fig. 44

(7) The Effect of Multiple MK-801 Injections on HSP72-li in the ARCN

We investigated whether the daily treatment regime of MK-801 which delays puberty (0.1 mg/kg from P27 to 32), affects HSP72-li in the ARCN/ME, retrosplenial cortex or cingulate cortex. MK-801 treatment for the first or the sixth time on P32 did not induce HSP72-li in any of these brain areas (not shown) at 12 or 24 hrs. after treatment. Control rats treated with saline did not exhibit HSP72-li.

(8) The Effect of Neonatal MSG on NMDA-Induced HSP72-li in the ARCN

Female rats treated neonatally with saline or with one of two MSG treatment schedules, were administered NMDA (20 mg/kg) on P28 and processed for HSP72-li. The first MSG treatment involved a single injection of MSG (2 mg/g) on P2 (experimental paradigm which induces early puberty; MSG (P2)). The second treatment involved an injection of MSG (4 mg/g) on P2, P4, P6 and P8 (experimental paradigm causing extensive neuronal damage and endocrinopathies (Bakke et al., 1978); MSG (P2 to P8)). Twelve hrs. post-NMDA on P28, the HSP72 response was similar in rats that had received either saline (Fig. 45A) or MSG (2 mg/g; Fig. 45B) on P2. In contrast, to this, very little HSP72-li was observed in the ARCN/ME of rats given the large dose (4 mg/g on P2, P4, P6 and P8) of MSG as neonates (Fig. 45C). Note that there are several widely dispersed HSP72-positive cells in the ventromedial hypothalamus (VMH) of the MSG (P2-P8) group which are absent in the other two groups (see arrowheads Fig. 45C). Saline treatment on P28 did not induce HSP72-li in the ARCN of either group (not shown).

Figure 45. - The effect of neonatal MSG treatment on NMDA-induced HSP72-li in the ARCN of juvenile female rats.

Photomicrographs of HSP72-li in the ARCN of 3 groups of P28 female rats 12 hrs. after administration of NMDA (20 mg/kg; s.c.). In A, rats were treated neonatally with saline (0.1 ml; s.c.) on P2. In B, rats were treated with MSG (2 mg/g; s.c.) on P2. In C, rats were treated with MSG (4 mg/g; s.c.) on P2, P4, P6 and P8. Scale bar = 100 μm. Note that C is off-centre to show the HSP72-positive cells which seem to be laterally dislocated.

A Saline Control

B Neonatal MSG P2

C Neonatal MSG P2-P8
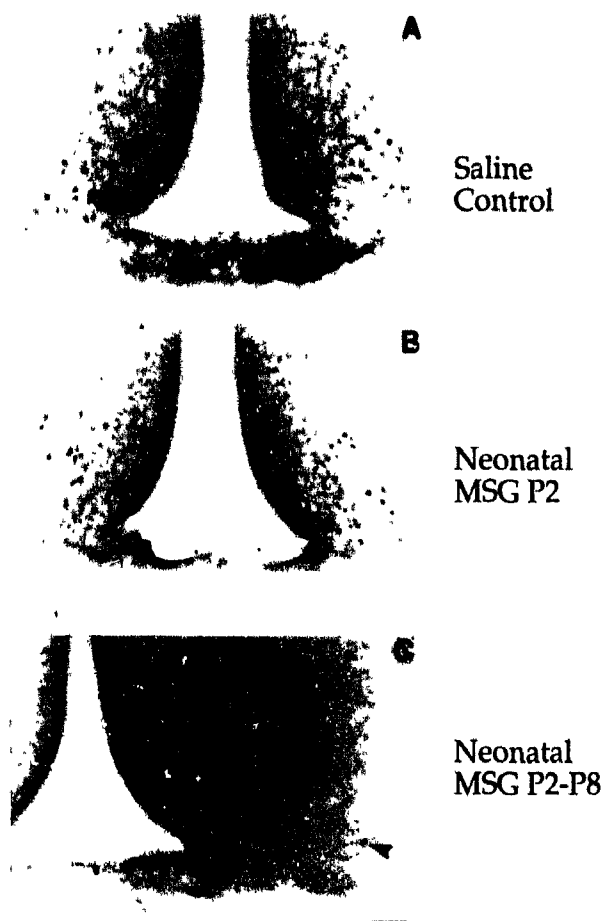
Fig. 45

(9) The Effect of other Excitatory Amino Acids on HSP72-li in the CNS

Kainic acid (KA; 10 mg/kg) but not saline treatment of juvenile female rats causes convulsions and induces a dramatic increase in HSP72-li in many areas of the CNS. Figure 46 illustrates the effects of KA on HSP72-li in the hippocampus, mediobasal hypothalamus, thalamus, perirhinal cortex and anteriolateral amygdala of prepubertal female rats 12 and 18 hrs. after treatment. KA induces intense HSP72-li in cells and fibres of the hippocampus of prepubertal female rats with the greatest immunoreactivity observed at the 18 hr. time point (Fig. 46F). The CA3 and the CA1 region contain HSP72-li but immunostaining is conspicuously absent from the CA2 region of the hippocampus (see arrowheads Fig. 46A, F). KA has a minimal effect on HSP72-li in the ARCN/ME but a small cluster of cells was found at the 12 hr. (see arrowhead Fig. 46B) but not the 18 hr. (Fig. 46G) time point in the rete hypothalamic nucleus. Many HSP 72-li cells and processes are found in the thalamus (Fig. 46E and 46J) and the perirhinal cortex (Fig. 46C and 46H) with the greatest immunoreactivity observed 12 hr. post-KA treatment. In addition, the anteriolateral (Fig. 46D and 46I) and the basolateral (not shown) amygdala contain HSP72-positive cells and the time course of expression appears similar to that observed in the thalamus and perirhinal cortex. Interestingly, a lower dose of KA (8 mg/kg) had either a dramatic or negligible effect on HSP72-li in all areas of the CNS (not shown). This all-or-nothing effect appeared to be related to the generation of seizure activity in these animals.

Figure 46. - The induction of HSP72-li in the CNS of juvenile female rats by kainic acid.

Kainic acid-induced HSP72-li in the CNS of P28 female rats 12 and 18 hrs. after injection (10 mg/kg; s.c.). HSP72 immunostaining is evident in the CA1 and CA3 regions of the hippocampus at both time points with the most dense staining seen at the 18 hr. time point (A and F). Very little HSP72-li was evident in the MBH (B and G) with the exception of the rete nuclei at 12 hrs. post-injection. Staining was also observed at both time points in the perirhinal cortex (C and H), amygdala (D and I) and thalamus (E and J) with the most intense staining seen at 12 hrs. Scale bar = 100 μm.

12 hrs.   18 hrs.
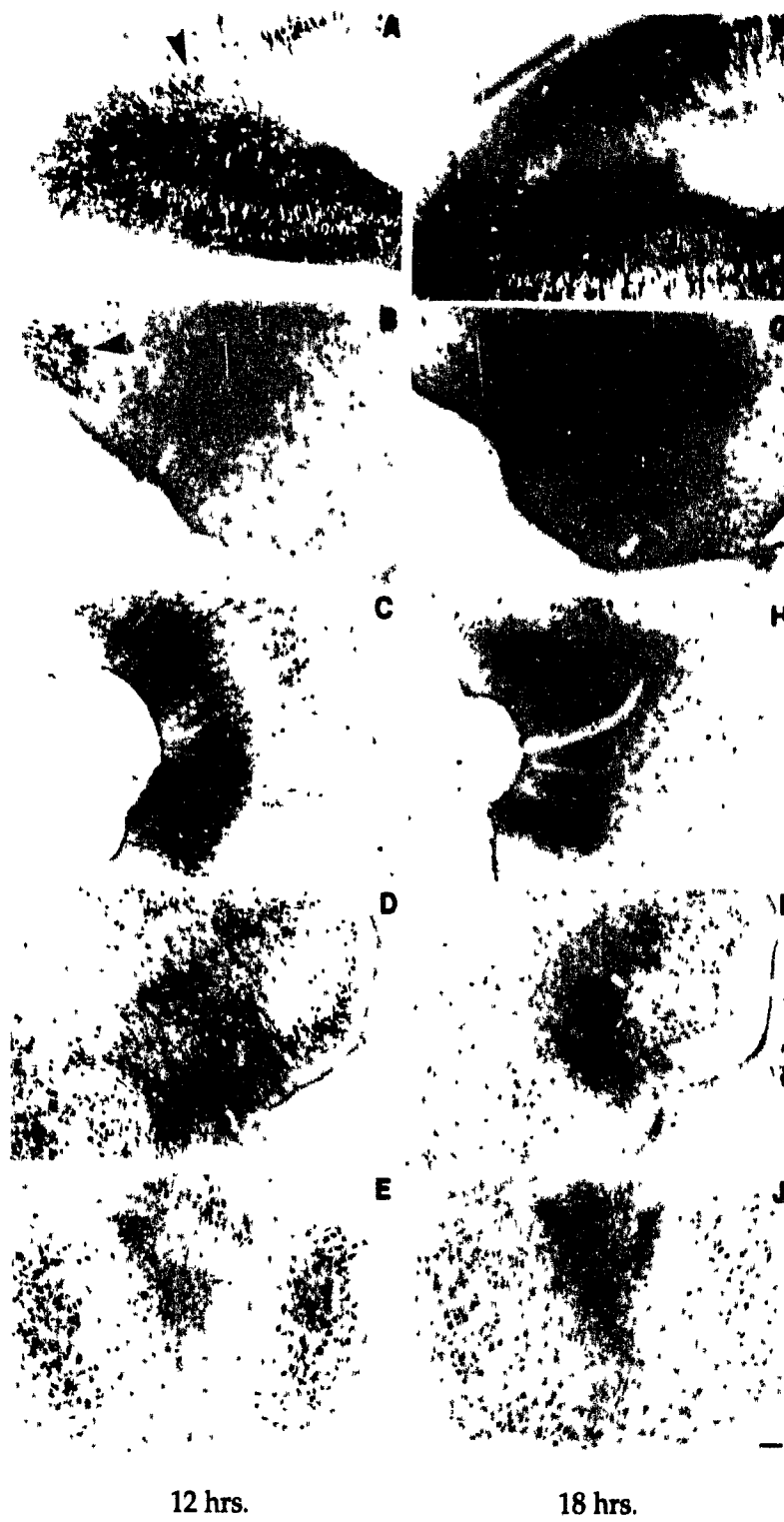
Fig. 46

Discussion

We have shown that groups of cells in the peripubertal female rat brain express the inducible stress protein HSP72 in a dose-dependent manner following systemic treatment with the glutamate agonist NMDA. Our studies are novel in that the only article published to date examining the relationship between NMDA and HSP used stereotaxic infusion of the drug into the EC of adult male rats (Yee et al., 1993). With the exception of Gonzales et al. (1989) very little work has been done on the effect of excitatory amino acids on HSP expression in the CNS. We confirmed using our immunocytochemical techniques the dramatic result of KA-induced "Golgi-like" HSP72-li in the hippocampus and amygdala reported by Gonzales et al. (1989).

The expression of HSP72 is thought to be a good indicator of cell injury in the CNS (see 'HSP and Neural Injury' in Introduction to section 3). As such, the induction of HSP72-li by NMDA appears to be somewhat incongruous with the postulation that NMDA is not neurotoxic to juvenile rats (Price et al., 1978a). They reported that doses of NMA less than 50 mg/kg (approximately 25 mg/kg of NMDA) do not induce pathological changes in the ARCN of P25 male rats. In addition, they showed that LH secretion is **not** decremental following consecutive daily injections of NMA (33 mg/kg) as expected if the NMA were actually toxic. In contrast, we reported that LH secretion is potentiated in the female rat following three consecutive daily injections of NMDA (20 mg/kg; s.c.) which would suggest that NMDA treatment is not toxic to our female rats either. It is possible that the NMDA-induced expression of HSP72 may have nothing do with 'stress' and cell death in our model of puberty. Although we were not able to show EB-induced HSP72 expression, Olazabal et al. (1992a, b) have reported that EB induces HSP70-li in the CNS of ovariectomized rats. The stress involved with physical restraint, which is known to activate the hypothalamic-pituitary-

adrenal axis, also increases HSP70 expression in adrenal cortex and aortic smooth muscle (Blake et al., 1991; Udelsman et al., 1993). Blake et al. (1991) stated that "these results suggest that not only do HSPs function under conditions of extreme stress but that they also play a fundamental role in maintaining homeostasis." Although the appearance of HSP72-li following NMDA treatment is highly suggestive of neuronal stress it may simply indicate the activation of an unknown physiological response.

We chose to examine HSP72 expression in circumventricular structures of the brain based on the identification of fos-like immunoreactivity (FLI) in these areas immediately following NMDA treatment. As discussed previously (see (1) under "Neonatal" and (1) under "Juvenile" Experiments in Chapter 3 Results), FLI can be detected in our peripubertal female rats in the ARCN, AP, SFO, AMPOA, PVN, and SON two hrs. post-NMDA treatment. HSP72-li was not observed in all the FLI-positive areas but was localized principally to the ARCN with minimal activation in the AP and SFO. The occasional HSP72-positive cell was evident in the AMPOA. As seen with c-fos immunostaining, the pattern of HSP72-li following NMDA treatment closely follows the boundaries of the ARCN from the most rostral to caudal sections. Other groups have reported the co-induction of immediate early genes (IEGs) and HSP72 in the brain following kainic acid treatment and hypoxia-ischemia (Schreiber et al., 1993a; Munell et al., 1994). Although c-fos and HSP72 expression co-localize following treatment, far fewer cells express HSP72-li relative to FLI suggesting that there may be a subgroup of cells which are sensitive to NMDA stimulation. Our results are in agreement with Munell et al. (1994) in a model of hypoxia-ischemia that HSP72 expression is absent from a number of anatomical structures that express c-fos and c-jun.

The localization of HSP72 expression in the NMDA-treated rats, parallels the neuronal structures previously shown to be vulnerable to glutamate

treatment (Olney, 1971; Price et al., 1978a; Seress, 1982). As discussed in the introduction, there is a large literature which sustains the opinion that HSPs are markers of stressed or damaged cells in the brain (for reviews see Brown, 1990; Sharp et al., 1993; Sharp and Sagar, 1994) but there are several exceptions to this rule (see "Neuronal Injury and Absence of HSP response" in Introduction to Chapter 4). The induction of robust HSP72-li in the ARCN and weak HSP72-li in the other CVOs, may demarcate those cells which have been differentially "stressed" by the injection of NMDA. However, there could also be a group of cells which have been injured by the NMDA treatment that are not expressing the protein. It would be useful to compare the immunocytochemical identification of HSP72 with evidence of cell damage following systemic treatment with NMDA. Initially, it would be beneficial to examine neuronal architecture with a Nissl stain. Several methods including Olmos's cupric-silver technique, acid fuschin staining (Auer et al., 1984), immunocytochemistry for p53 (Chopp et al., 1992) and a recently developed method using a photo oxidized fluorescein derivate (Fluoro-Jade-D; Schmued et al., 1993) could be used to detect neuronal degeneration.

Immunostaining in P28 female rats in the ARCN is not apparent until 4 hrs. post-injection, is maximal between 8 and 12 hr. and then gradually declines in intensity between 1 and 7 days after NMDA. Yee et al. (1993) reported that HSP72 expression following intracerebral injections of NMDA peaks slightly later (18-48 hr.) than we observed. This may be due to differences in the control of gene transcription in EC compared to ARCN, or due to the trauma associated with drug delivery or the age/sex of the rats.

Long-term expression (days) of HSP72-li in our NMDA-treated animals may be indicative of permanent neuronal damage. Schreiber et al. (1993a) have noted that transient expression (4 to 8 hrs.) of HSP72 occurs in neurons resistant

to systemic KA treatment (10 mg/kg; s.c.) (dentate granule cells, parietal cortical cells) but prolonged expression (>16 hrs.) is observed only in vulnerable neurons (CA1 and CA3 pyramidal cells, and neurons of thalamus, amygdala and piriform cortex). Tomioka et al. (1993) have also reported the prolonged expression of HSP72 in vulnerable areas of the hippocampus following transient ischemia in the rat. A similar correlation between prolonged c-fos expression and neuronal death following KA treatment has been reported by Smeyne et al. (1993). Fos-lacZ is evident in the vulnerable CA1 and CA3 regions of the hippocampus of transgenic mice 7 days after KA injection (17 mg/kg; i.p.).

The time course of the appearance of HSP72-li in the ARCN after the fifth daily injection of NMDA is very similar to that seen after the first injection. However, the intensity of the stain and the number of HSP72 immunopositive cells is increased in the NMDA (x5) group relative to NMDA (x1) at 4, 8 and possibly 24 hrs. post-injection. The increased immunoreactivity may be due to residual HSP72 from previous injections. Alternatively, daily treatment with NMDA may have a cumulative effect on cell stress, triggering a proportionate and rapid increase in HSP72 protein synthesis. The apparent 'additive' effect of daily NMDA treatment on HSP72-li is contrary to the decline in FLI that I described in Chapter 3 (see experiment (4) under Juvenile Experiments in Results of Section 2) following daily NMDA treatment. Based on the c-fos data, I hypothesized that NMDA receptors may be down-regulated by the daily treatment regime. The HSP72 results seem to contradict this hypothesis but the difference in the c-fos and HSP72 response to repetitive injections of NMDA may be accounted for by differences in the half-life of the proteins and accumulation of HSP72 as opposed to rapid degradation of fos.

The only published heat shock studies that are analogous to the repetitive treatment in our experiments examined ischemic tolerance brought about by

preconditioning ischemia (see section on 'HSP and Neuronal Protection' in Introduction to section 3). Liu et al. (1993) reported that compared to sham treatment, preconditioning rats with sublethal ischemia (3 min.) protected them from neuronal damage following a subsequent lethal period of ischemia (6 min.). Two hrs. after the second ischemic episode, HSP72-li was observed in preconditioned, but not in sham animals. One and three days after ischemia, HSP72-li was equivalent in sham and preconditioned animals. Interestingly, at seven days post-ischemia, the sham group expressed very little HSP72 whereas the immunostaining in the preconditioned group remained moderately elevated. The prolonged expression of HSP72 reported by Liu et al. (1993) was not observed in our experiments following the fifth injection of NMDA. The differential effect of preconditioning ischemia a d daily injections of NMDA on HSP72 cannot be explained especially considering that prolonged expression of HSP72 appears to be associated with neuronal death (see above).

Treatment of neonatal rats with NMDA or MSG does not induce HSP72-li in the mediobasal hypothalamus (MBH). A heat shock response is not apparent until P15, is maximal between P21 and P28 and then declines to adult levels. This is an interesting result as we know from previous publications (Olney, 1971; Olney and Price, 1978; Seress, 1982) that neonatal MSG significantly damages the ARCN yet the stress protein indicating cellular injury is apparently not expressed or detectable with this antibody. Cells in the ARCN that are damaged by MSG may fail to express HSP72 due to a transcription or translation block. The absence of HSP72-li in damaged tissue is similar to that observed by Kinouchi et al. (1993) at the centre of the forebrain lesion caused by MCA occlusion. Alternatively, HSP72-li in our experiments may be obscured from view due to cellular edema. Recall that FLI is localized to an arc of tissue over an empty dome-shaped area in the hypothalamus following MSG (0.25 to 4 mg/g; see

neonatal experiment (2) in Results of Section 2). This empty area has also been observed in brain sections from domoic acid-treated rats (Bruni et al., 1991). The cellular edema hypothesis seems unlikely, however, as it is highly improbable that not one nucleus would be visible in the 100 or 50 μm section of tissue. One other possibility is that the expression of HSPs may be stimulus dependent i.e. ischemia, but not EAA, leads to an increase in HSP72-li in the ARCN.

Others have shown that the induction of HSP72, especially in rat brain, appears to depend on the stage of postnatal development. White (1981) examined HSP72 expression in organ slices in brain, heart and lung at different stages of development. Interestingly, HSP 72 expression was evident on P1 in heart and lung but is absent in brain until 2 weeks of age. A developmental study was conducted by Currie et al. (1983) using hyperthermia (42°C for 15 min.) as a perturbation. They reported the synthesis of SP71 (HSP72) in heart, lung, liver and kidney in newborn through 6 week old rats. However, the levels of HSP72 in shocked animals in brain tissue were undetectable until 3 wks. of age. Quite recently, it has been shown that KA stimulation c⁵ a heat shock response does not occur until P21 in the rat (Schreiber et al., 1993a). In contrast to these results, Brown (1983) used Northern blots to detect HSP70 mRNA in neonatal rat brain following exposure to a heat lamp. The immunocytochemical detection of HSP72 has also been reported in 1 wk. old rats (Ferriero et al., 1990) and HSP70 mRNA has been detected using Northern blot hybridization and *in situ* hybridization in forebrain of 1 wk. old rats following hypoxia-ischemia (Gubits et al., 1993; Munell et al., 1994). Again, the contrary results may be an indication that HSP72 expression is stimulus dependent or that more sensitive techniques such as polymerase chain reaction (PCR) may be required to detect the heat shock response.

A comparison of P15 and P21 at the 12 and 24 hr. time points shows that the area of distribution of HSP72-li is wider in the younger animals. The narrowing of the circumference of the area containing HSP-72 positive cells with age may relate to the maturation of the BBB in these animals which is ongoing through the 2nd and 3rd week of life (see Landymore and Wilkinson, 1990). Alternatively, though less likely, it may relate to the changing pattern and sensitivity of glutamate receptors in the hypothalamus (see Chapt. 1 Introduction on 'Developmental Expression of EAA and their Receptors').

NMDA-induced HSP72-li in the ARCN can be blocked by the administration of the noncompetitive NMDA receptor antagonist, MK-801, or the competitive antagonist, AP5. Pretreatment with MK-801 at 0.1 mg/kg only partially inhibited HPS72-li but expression was completely blocked with the 1 mg/kg dose. This is similar to the results reported for FLI (see experiment (2) in 'Juvenile Experiments' in Results of section 2) Similarly, APV pretreatment (20 mg/kg) completely blocked NMDA-induced HSP72-li confirming the involvement of NMDA receptors in the induction of HSP72-li.

In our hands, MK-801 treatment (0.1 mg/kg) of peripubertal female rats is not related to an increase in HSP72-li in the ARCN, nor in the retrosplenial (RC) or posterior cingulate cortex (PCC) as reported by Sharp et al. (1991a). This group showed that treatment of adult female rats with MK-801 (0.1 to 5 mg/kg; i.p.) induced HSP72-li in injured neurons of the RC and PCC with maximal staining observed with the 1 mg/kg dose. HSP72-positive neurons were evident between 18 hours and 7 days after treatment with the noncompetitive NMDA receptor antagonist. Variation in the sensitivity and density of NMDA receptors in the developing brain may explain the discrepancy between our results and those of Sharp et al. (1991a). Sharp's group uses an Amersham antibody which is very similar to that used in the present work (StressGen) in that it reacts mainly

with the inducible HSP70 protein. The MK-801 experiment should be repeated in peripubertal and adult female rats with a gradient of doses between 0.05 and 5 mg/kg in order to make a direct comparison with Sharp's study.

NMDA-induced HSP72-li in P28 rats is altered by neonatal treatment with high dose MSG (4 mg/g on P2, P4, P6 and P8) but not by low dose MSG (2 mg/g on P2) or saline. This result is reminiscent of experiments that demonstrated that the pattern of FLI in the ARCN after NMDA was completely different in rats treated neonatally with high dose MSG compared to those rats treated neonatally with saline or low dose MSG (MacDonald et al., 1990). Olney et al. (1971) have described the high dose MSG-induced destruction of close to 90% of the neurons in the arcuate nucleus followed by the ventral migration of the VMN into the damaged ARCN. The fact that the pattern of FLI and HSP72-li in the ARCN is largely unaltered by the low dose neonatal MSG treatment (used to reliably accelerate puberty) suggests that a single injection of MSG on P2 does not significantly eliminate NMDA-responsive neurons. In addition, MSG treated neonates have normal estrous cycles after puberty and do not exhibit the growth and endocrinological abnormalities observed in those rats treated neonatally with high dose MSG.

Contrary to an earlier report (Olazabal et al., 1992a, b), we did not observe an increase in HSP72 synthesis following treatment with estradiol benzoate (EB). Olazabal et al. (1992a) showed that within 12 hrs. of EB administration, both HSP70 and HSP90 protein concentrations increase in the ventromedial hypothalamus (VMH) of ovariectomized female rats. EB has no effect on HSP expression in castrated male rats (Olazabal et al., 1992a). The antibody used by this group recognizes both the constitutive and inducible forms of HSP70. If only HSC70 rises after EB, this would explain the lack of HSP72 (inducible)-li in our animals. This would be in agreement with the report of an estrogen-stimulated

increase in HSC70 mRNA in brain (Mobbs et al., 1989). Alternatively, ovariectomy may upregulate transcription of the estrogen receptor, thereby increasing the sensitivity to EB and promoting a detectable heat shock response. Our immunocytochemistry experiment should be repeated in ovariectomized prepubertal rats. Other work by this group showed that EB stimulated increases in HSP90 in VMH and uterus and HSP90-li in the brain appears to be entirely neuronal (Olazabal et al., 1992b). However, preliminary studies in our laboratory of EB-induced HSP90-li in the AMPOA and ARCN were negative (studies not included). Perhaps a better vehicle for examining the heat shock response to steroids may be the low molecular weight HSP27 which appears to be estrogen-inducible (Padwick et al., 1994).

The principal site of NMDA-induced HSP72-li is the ARCN as relatively little HSP72-li is detected in the SFO, AP or AMPOA. NMDA increases the expression of HSP72-li in the ARCN of juvenile female rats in a dose- and age-dependent manner which can be inhibited by the NMDA antagonists MK-801 and APV. In our model of NMDA-induced precocious puberty, repetitive injections may cause accumulation of HSP72 in cells of the ARCN. Although treatment with the glutamate analogue increases HSP72-li, the question is still open as to whether NMDA treatment of juvenile female rats causes neuronal damage. It is also possible that NMDA-induced HSP72-li is a physiological response of unknown function. For instance, Olazabal et al. (1992a,b) have demonstrated that EB increases HSP70 expression in the brain despite the fact that it is not a neurotoxin.

Obviously, the experiments on EAA-induced HSP72 generated more questions than they answered. Several lines of experimentation arise from this information.

(i) The results would be less subjective if an image analysis system was used to quantify the number of HSP72-positive cells in the ARCN of NMDA-treated animals.

(ii) What types of cells (neurons, glia, astrocytes) express HSP72 in response to NMDA treatment? What is their phenotype (GABA, opiates, adrenergic, neuropeptide Y)?

(iii) In our model of puberty, is HSP72 expression a marker of cell damage? To examine this other markers of cell damage such as acid-fuschin staining and Fluoro-Jade-D could be compared with HSP72-li following NMDA treatment.

(iv) *In situ* hybridization could be used to localize and quantify HSP72 mRNA following MSG or NMDA treatment. This would give information on the differential distribution of HSP72 mRNA and its protein perhaps indicating areas where translation is inhibited.

The hypothesis that neurons in the ARCN may be injured or stressed by systemic administration of NMDA is an attractive one. ARCN lesions have long been implicated in the early onset of sexual maturation (Ruf et al., 1975; De Ziegler et al., 1976). One current line of research is that lesion-induced precocity may be regulated by the induction of growth factors such as transforming growth factor α (TGFα) (Junier et al., 1991). In the last chapter of this thesis I have examined the possibility that NMDA treatment may alter the expression of another neurotrophic factor, basic fibroblast growth factor (bFGF).

CHAPTER 5 - NMDA-STIMULATED EXPRESSION OF bFGF

Introduction

NMDA treatment has a stimulatory influence on the attainment of reproductive competence in various animal models, including the rat. Its mechanism of action remains equivocal but, in general, may involve the removal of an inhibitory influence or the imposition of an excitatory drive (see Chapter 1). The induction of c-fos and HSP72 would be consistent with either hypothesis. However, an alternative postulate is that the glutamate agonist may enhance the excitatory drive to puberty by stimulating neurite outgrowth (i.e. see "synaptogenic hypothesis of puberty" in Introduction to chapter 1 for a summary of the experimental evidence linking hypothalamic connectivity and puberty). NMDA may act directly on synaptogenesis (McDonald and Johnston, 1990a; Constantine-Paton, 1990) or via an intermediary such as a neurotrophic factor. For instance, Zafra et al. (1990, 1991) have demonstrated that stimulation of glutamate receptor subtypes regulates the expression of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in hippocampal neurons both *in vivo* and *in vitro*. Hughes et al. (1993a) have recently shown that focal brain injury leads to a transient induction of BDNF mRNA via NMDA receptor activation. In addition, Humpel et al. (1993) reported an increase in bFGF and its mRNA in the forebrain and hippocampus of rats treated with kainic acid. These data suggest that EAAs may regulate cell differentiation and neurite outgrowth in various regions of the brain, possibly the hypothalamus, by triggering the expression of neurotrophic factors.

We hypothesize that the role of NMDA in exciting precocious sexual maturation may be mediated, at least partially, through growth factors.

I. Growth Factors and Sexual Maturation

There is a growing body of evidence implicating neurotrophic factors in the maturation of hypothalamic neurons and the onset of puberty (for review see Ojeda, 1992). The POA and MBH of female rats express the highest quantities of NGF and NGF.R mRNA in the first two weeks of postnatal life (Ojeda et al., 1992). The genes encoding transforming growth factor α (TGFα) are also expressed at high levels in the prepubertal female hypothalamus with peak levels at P12, declining by P23 and rising briefly on the afternoon of first proestrus (Ojeda et al., 1990). In the ARCN/ME area, transcription of epidermal growth factor receptor (EGF.R) declines on the morning of proestrus and rebounds at the time of the LH surge (Ma et al., 1994b). Similarly, Michels and Saavedra (1991) have identified a peak of insulin-like growth factor I (IGF-1) binding in the ME of hamster hypothalamus on P15. The developmental regulation and high concentration of NGF, TGFα and IGF-I and their respective receptors in the hypothalamus and POA of rats suggests a potential role for growth factors in the regulation of sexual maturation (Levi-Montalcini et al., 1990).

Lesions of the hypothalamus are known to induce precocious puberty (Donovan and van der Werff ten Bosch, 1965). More recently, TGFα has been directly implicated in this phenomenon (Junier et al., 1991). Radiofrequency lesions of the preoptic-anterior hypothalamic area induce precocious sexual maturation and result in a significant increase in TGFα mRNA, TGFα-precursor-li, EGF.R mRNA and EGF.R around the site of the lesion (Junier et al., 1991, 1993; Ma et al., 1994a). Lesion-induced early puberty is inhibited by stereotaxic infusion of RG-50864, a blocker of EGF/TGFα-tyrosine kinase activity. Similarly, implantation of RG-50864 in the median eminence (ME) of intact female rats significantly delays puberty (Ma et al., 1992). These studies strongly suggest that

TGFα is an important component of the neuroendocrine mechanism controlling the onset of puberty.

Growth factors in the mammalian nervous system may also be involved in neuroendocrine secretory processes. Several growth factors (EGF, TGFα and bFGF) stimulate a dose-related increase in LHRH release from nerve terminals in the MBH (Ojeda et al., 1990). Similarly, IGF-I, IGF-II and insulin stimulate LHRH release from the ME of juvenile female rats (Hilney et al., 1991). Interestingly, estradiol upregulates the expression of TGFα mRNA in hypothalamic but not cerebellar astrocyte cultures (Ma et al., 1994a).

TGFα and the NGF receptor (NGF.R) have been examined by this laboratory for a possible role in NMDA-mediated precocious puberty but the most interesting area of research appears to be basic fibroblast growth factor (bFGF). **This chapter will focus on the role of bFGF and its receptor in NMDA-mediated early puberty.**

II. Basic Fibroblast Growth Factor

The fibroblast growth factors, bFGF and acidic FGF (aFGF), belong to a family of closely related heparin-binding polypeptides with diverse biological activities (Burgess and Maciag, 1989). There are 7 members in the heparin-binding growth factor (HBGF) family and they include: acidic FGF (FGF-1), basic FGF (FGF-2), INT-2 (FGF-3), ks-FGF/hst-1 (FGF-4), FGF-5, hst-2 (FGF-6) and keratinocyte growth factor (Baird and Bohlen, 1990). The members of the HBGF family are either growth factors or the product of a proto-oncogene and exhibit 45 to 60% homology in amino acid composition.

Structural analysis has shown that bFGF is a single chain polypeptide composed of 146 amino acids with a molecular weight of 16,000. It can also exist as a NH_2-terminally truncated form, missing the first 15 amino acids (des.1-15

bFGF). bFGF has two binding domains for heparin, one near the NH_2-terminal region and one near the COOH terminus (Gospodarowicz et al., 1987). Interestingly, bFGF lacks a signal peptide and it is unknown how the protein is secreted from the cell. The bFGF gene is at least 36 kb in length and consists of three exons and two introns (Shibata et al., 1991). In rat, the gene is transcribed into two mRNAs of approximately 3.7 and 7 kilobases (kb).

III. Basic Fibroblast Growth Factor Receptor

Four FGF receptor subtypes have been identified and they include FGF.R1 or *flg*, FGF.R2 or *bek*, FGF.R3 or *cek2* and FGF.R4 (Horlick et al., 1992). The receptors in this family have several common characteristics: a hydrophobic sequence, an acid binding region, immunoglobulin-like domains, hydrophobic transmembrane domain and a tyrosine kinase domain. The transduction of the FGF signal is initiated by FGF binding to its cell membrane receptor leading to the tyrosine phosphorylation of phospholipase C-II. The activated enzyme initiates the breakdown of phosphatidyl inositols and the release of diacylglycerol which activates protein kinase C. A few FGF receptors are produced as soluble secreted forms rather than tyrosine kinase linked moieties. Dionne et al. (1991) suggest that the secreted FGF receptors may bind FGF and downregulate its signal or transport FGF to an extracellular site of action.

IV. Localization of bFGF, its Receptor and Gene Expression in the CNS

Basic FGF has been isolated from a wide range of mesoderm- and neuroectoderm-derived tissues, including: brain, pituitary, retina, corpus luteum, adrenal gland, kidney, placenta, macrophages, prostate, thymus, bone and various tumours (reviewed in Gospodarowicz et al., 1986a,b). Basic FGF-like immunoreactivity (bFGF-li) has also been identified in several important nuclei in the neuroendocrine axis including: dorsomedial hypothalamic nucleus

(DMN), POA, mammillary nucleus, PVN, SON, SFO with the highest density in the VMN (Iwata et al., 1991; Frautschy et al., 1991; Matsuyama et al. 1992).

There are conflicting reports over the cellular localization of bFGF-li in rat brain. FGF-li has been reported to exist primarily in the cytoplasm and cell processes of neurons and to have a ubiquitous distribution through the tissue (Pettman et al., 1986). Although Matsuyama et al. (1992) reported that bFGF-li is regionally localized, immunopositive neurons were found in the cerebral cortex, olfactory bulb, thalamus, hippocampus, amygdala, central gray of the midbrain, cerebellum, spinal cord, hypothalamus and several other areas of the brain. Contrary to these findings, other groups have used double-staining immunohistochemistry to identify astrocytes as the main locus of bFGF-li with minor immunocytochemical staining in specific neuronal populations of the adult rat (Gomez-Pinilla et al., 1992, 1994; Gomez-Pinilla and Cotman, 1992; Woodward et al., 1992). In support of these findings, bFGF-li is found in purified cultures of astrocytes but not neurons from the cerebral cortex (Woodward et al., 1992). The discrepancy in the distribution of bFGF may be explained by the difficulty in making specific antibodies to individual members of the FGF family due to their structural homology. Furthermore, and in contrast to the other groups, the studies of Pettmann et al. (1986) were performed in neonatal and juvenile rats and they describe a decrease in neuronal bFGF-li with aging. Indeed, Gomez-Pinilla et al. (1994) have recently reported that "the cellular location of basic fibroblast growth factor changes during development..." from being primarily neuronal in embryonic and neonatal rats to astrocytic from P20 to adults.

It has been reported that the brain is the only normal adult tissue which expresses detectable levels of bFGF mRNA (Abraham et al., 1986a,b; Shimasaki et al., 1988; Emoto et al., 1989). Using Northern blot hybridization, 3 bFGF mRNA

transcripts (6.0 (principal), 3.0 and 1.6 kb) were detected in equivalent amounts of RNA from the hypothalamus, frontal cortex, parietal cortex, occipital cortex, hippocampus and pons with very little expressed in the cerebellum or pituitary (Emoto et al., 1989). The same group examined the RNA from purified cultures of neurons and astrocytes from the entire telencephalon of fetal (E18) rats and only the purified glial cultures expressed bFGF mRNA. bFGF mRNA has also been identified in rat brain using polymerase chain reaction (PCR) (Shimojo et al., 1991) and *in situ* hybridization (Emoto et al., 1989) techniques.

In situ hybridization has also been used to localize FGF.R mRNA in the rat CNS (Wanaka et al., 1990). The hybridization signal was widely distributed in the brain with focal areas of intensity and appeared to belong to neuronal populations. Interestingly, the intensity of the labeling was very strong in the median eminence, strong to moderate in the circumventricular organs (SFO, AP, OVLT) and moderate to weak in the arcuate nucle. s.

There is evidence indicating that bFGF expression is regulated during development (Caday et al., 1990; Riva and Mocchetti, 1991; Giordano et al., 1991). Riva and Mocchetti (1991) using an RNase protection assay reported low levels of bFGF mRNA in neonatal rats which rise to maximal levels at P21. Similar results were reported by Caday et al. (1990) using immunoblotting and a bioassay of mitogenic activity. In addition, Giordano et al. (1991) reported a variation in the developmental pattern of expression of four molecular weight forms of bFGF (18, 21, 22, 24 kDa) using Western blots suggesting that the individual forms of bFGF may have novel functions in the CNS.

V. Neurotrophic Effects of bFGF

bFGF is a potent mitogen and can act as a cell differentiation and survival factor in a wide range of tissues, including the brain (Gospodarowicz et al.,

1986a). Indeed, bFGF enhances the survival of cultured hippocampal (Walicke et al., 1986; Matsuda et al., 1990), cortical (Morrison et al., 1986, Walicke, 1988), ciliary ganglion (Schubert et al., 1987) striatal (Walicke, 1988; Matsuda et al., 1990), septal, (Grothe et al., 1989; Knusel et al., 1990; Matsuda et al., 1990), mesencephalic dopaminergic (Knusel et al., 1990; Beck et al., 1993), and anterior thalamic (Walicke, 1988) neurons. bFGF also promotes neurite extension in cultured hippocampal (Walicke et al., 1986; Walicke, 1988), cortical (Morrison et al., 1986; Walicke, 1988), striatal neurons (Walicke, 1988) and PC-12 cells (Wagner and D'Amore, 1986; Rydel and Greene, 1987). Neuronal cultures derived from the cerebellum (Morrison et al., 1988) or the subiculum (Walicke, 1988) do not respond to bFGF with enhanced survival or neurite extension.

Most relevant to this thesis, bFGF is reported to have neurotrophic effects on hypothalamic cell cultures (Torres-Aleman et al., 1990; Ishikawa et al., 1992). bFGF induces a dose-dependent increase in neurite-bearing cells and a 2.5 fold increase in glial fibrillary acidic protein-immunoreactive cells in primary hypothalamic cultures (Torres-Aleman et al., 1990). In addition, bFGF significantly increased the number of surviving neurons in neuron-enriched cultures. Similar results were obtained by Ishikawa et al. (1992) for growth hormone releasing factor- , somatostatin- and vasoactive intestinal peptide-li neurons in cultured hypothalamic neurons. Torres-Aleman et al. (1990) used [^{3}H]-thymidine incorporation as a measure of mitotic activity and reported that bFGF stimulated cell proliferation in enriched glial cultures. Similarly, bFGF increased the growth rate of two SV-40 transformed hypothalamic cell lines (A-6 and F-12) (Torres-Aleman et al., 1990). bFGF and IGF-I have additive effects on the number of neurite bearing cells surviving in mixed hypothalamic culture suggesting a coordinated trophic action (Torres-Aleman et al., 1990). bFGF increases the number of IGF-I receptors and causes a dose dependent release of

IGF-I into the medium of cultured hypothalamic cells (Pons and Torres-Aleman, 1992).

In addition to its neurotrophic effects, bFGF is also a mitogen for astrocytes (Pettman et al., 1985; Walicke and Baird, 1988; Morrison et al., 1988; Torres-Aleman et al., 1990), oligodendrocytes (Eccelston and Silberberg, 1985; Saneto and De Vellis, 1985) and Schwann cells (Pruss et al., 1981).

VI. Neuroprotective Effects of bFGF

Trophic factors such as bFGF, may not only participate in the development and maintenance of the normal cytoarchitecture of the brain, but also in its response to injury. The healing processes that are activated by neuronal damage include angiogenesis, absorption of neuronal debris by phagocytic macrophages from the general circulation, proliferation of glial cells (astrocytes and microglia) (Kelly, 1981) and sprouting of neurons at the borders of injury sites (Purves and Lichtman, 1985). These responses to CNS injury inhibit subsequent damage, repair damaged tissue and may contribute to the recovery of neuronal function following CNS injury. Neurotrophic factors may participate in the response to injury by preventing neuronal degeneration and promoting neurite outgrowth.

A number of injuries (aspiration, knife wound, axotomy, electrolytic ablation, ischemia) have been found to increase bFGF expression in different areas of the CNS (Finkelstein et al., 1988; Gomez-Pinilla et al., 1992; Logan et al., 1992a,b; Gomez-Pinilla and Cotman, 1992). Gomez-Pinilla and Cotman (1992) suggest that the "injury-induced expression of bFGF and its receptor may be related to a mechanism of self-protection and/or plasticity". Several recent articles have shown that endogenous and exogenous bFGF can prevent neuronal death in damaged or axotomized areas of the brain. Otto et al. (1989) reported

that implantation of gel foam soaked with bFGF or NGF reduced cholinergic neuronal loss in the medial septum (MS) 30 days following transection of the fimbria-fornix pathway. Similar results were reported by Anderson et al. (1988) in the MS and the vertical limb of the diagonal band of Broca (vDB) in adult (3-4 mos.) and to a lesser extent in aged (24-26 mos.) rats. bFGF rescued cell death has also been reported in the peripheral nervous system (sympathoadrenal preganglionic neurons) following adrenomedullectomy (Blottner and Baumgarten, 1992).

Basic FGF can also protect neurons from NMDA.R mediated excitotoxicity. Glutamate (50 µM to 1mM) added to cultures of hippocampal neurons reduces dendritic and axonal outgrowth and also reduces neuronal survival in a dose dependent manner, but if bFGF is present, the threshold of glutamate induced neurotoxicity is significantly increased (Mattson et al., 1989; Skaper et al., 1993). The neuroprotective effects of bFGF are also evident in cultured striatal neurons exposed to glutamic acid (3 mM) and quinolinic acid (1mM). However, Freese et al. (1992) reported that bFGF did not prevent the destruction of striatal neurons following kainic acid (1mM) exposure. The ability of bFGF to block EAA associated neurotoxicity is also evident *in vivo* (Nozaki et al, 1993a,b; Frim et al., 1993). Administration (i.p.) of bFGF 30 min. prior to or 2 hr. after an intrastriatal injection of NMDA to neonatal rats (P7) significantly reduces neuronal damage (Nozaki et al., 1993a). Pretreatment with bFGF also diminished toxicity induced by hypoxia-ischemia in a dose dependent manner (Nozaki et al., 1993b). Similarly, neural implantation of genetically engineered fibroblast cell lines which secrete bFGF or NGF significantly reduces (by 30% and 80%, respectively) the lesion produced by stereotaxic infusion of quinolinic acid (NMDA agonist) into the striatum (Frim et al., 1993).

VII. Identification and Measurement of bFGF Expression

A relationship between the NMDA receptor and bFGF in our model of sexual maturation is an exciting possibility. This hypothesis is based on the hypothalamic distribution of bFGF, its hormonal influence on LHRH and the complementary relationship between bFGF and NMDA in neurotoxicity/neural architecture. The question then becomes, how do we quantify bFGF expression in the hypothalamus? We chose to examine the role of bFGF expression in the onset of puberty using immunocytochemistry and a sensitive technique called **polymerase chain reaction (PCR).** Immunocytochemistry is well established in our laboratory but the molecular biology experiments in this chapter of my thesis represent preliminary work using new techniques to our laboratory which have been applied with much assistance from Dr. Paul Murphy and his graduate student, Rai Knee.

PCR can be used to detect extremely small concentrations of a gene from a complex mixture of DNA by replicating small segments of the selected gene. The crucial ingredient in a PCR reaction is an enzyme called Taq polymerase. It is derived from a bacterium (*Thermus aquaticus*) indigenous to hot springs and ocean vents, and catalyzes the synthesis of new DNA at temperatures which would denature most proteins. The first step of the PCR reaction involves heating the experimental sample to separate the DNA into 2 strands. The temperature is lowered slightly, allowing specific complementary oligonucleotide probes (DNA primers) to anneal to the analogous sequence on each strand. Next, the temperature is increased slightly, and Taq polymerase drives the incorporation of deoxyribonucleotide triphosphates (dNTPs) into each new strand. The reaction is halted by increasing the temperature and separating the newly formed strands from their DNA template, effectively doubling the

DNA with each cycle. The cycle can be repeated many times over and leads to the exponential production of DNA (see Figure 47).
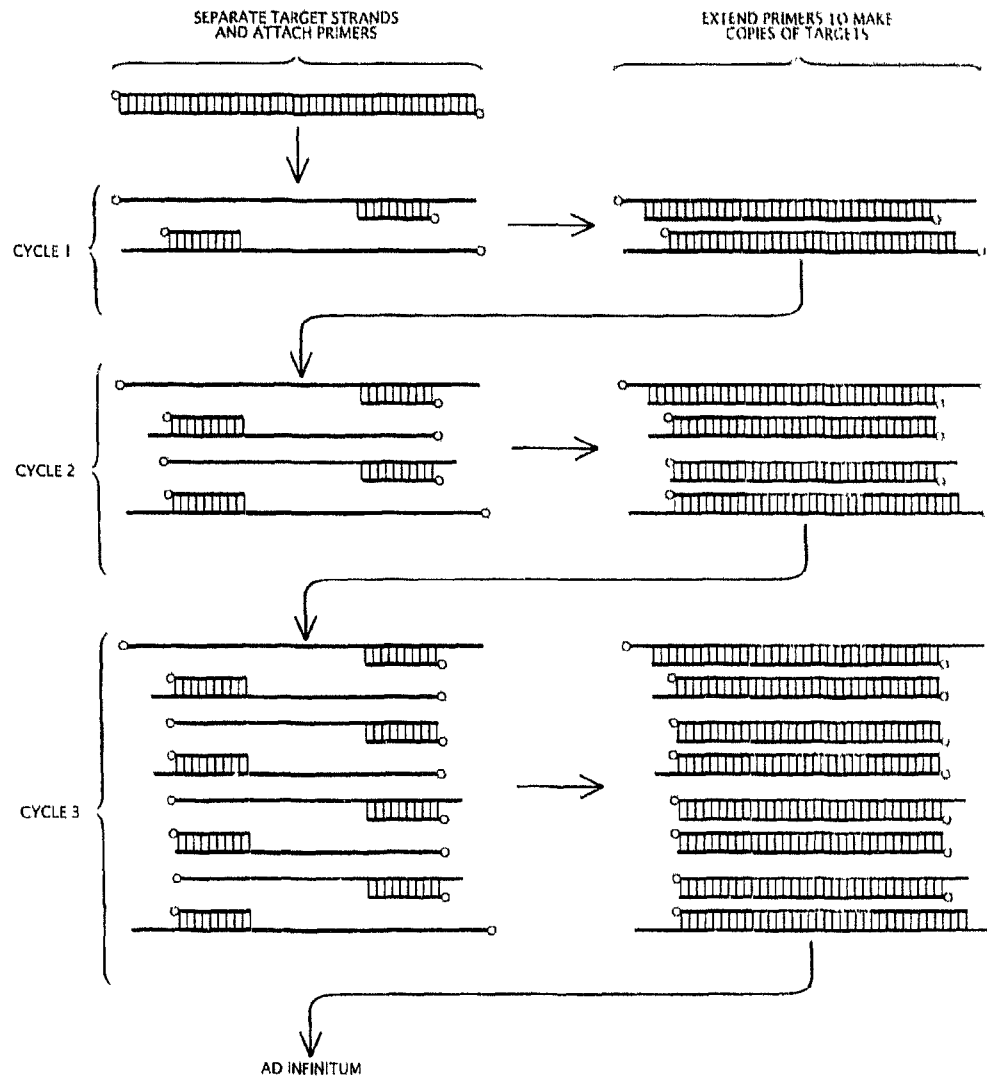
Fig. 47 - The process of polymerase chain reaction.

Polymerase chain reaction is a cyclic process; with each cycle, the number of DNA targets doubles. The strands in each targeted DNA duplex are separated by heating and then cooled to allow primers to bind to them. Next DNA polymerases extend the primers by adding nucleotides to them. In this way, duplicates of the original DNA-strand targets are produced. (Redrawn from Mullis, 1990).

There are a few characteristics which make PCR a difficult tool to use for quantification. For instance, minor differences in DNA concentrations in primary samples will be exponentially increased with each cycle. In addition, as the cycle number increases, the production of new DNA product will decline or plateau as the dNTPs are depleted. However, these technical difficulties can be overcome by characterizing the reaction and establishing the optimal point (i.e. cycle number) for examining the PCR product.

The experiments in this section were part of an evolving process whereby the dynamics of the technique were characterized and modified over time. The work does not represent a complete analysis of the role of bFGF in NMDA-mediated precocious puberty but does identify some link between NMDA and bFGF and generates many ideas for continuing this line of research.

Materials and Methods

I. Chemicals

Deoxynucleotide triphosphates (dNTPs) and random hexanucleotide primers (pd(N)$_6$) were purchased from Pharmacia Fine Chemicals, Dorval, Quebec, Canada. Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase, restriction endonucleases and dithiothreitol (DTT) were obtained from BRL/Life Technologies, Burlington, Ontario, Canada. Taq DNA polymerase and RNasin were from Promega, Madison, WI. Recombinant human bFGF was a gift from Takeda Chemical Industry (Osaka, Japan). Pregnant mare's serum gonadotropin (PMSG) and, unless otherwise noted, all other chemicals were from Sigma Chemical Company, St. Louis, Mo.

II. Animals

As described in Chapters 2 to 4.

III. Oligodeoxynucleotides

Oligodeoxynucleotide primers for PCR were synthesized at the Marine Gene Probe Laboratory, Dalhousie University, Halifax, Nova Scotia, Canada. The PCR primer sequences for bFGF and FGF receptor have been previously used to amplify reverse transcribed human RNA (Murphy et al., 1992). However, the sequences are well conserved between species and span defined intron-exon boundaries to permit discrimination between cDNA and possible contaminating genomic DNA. The bFGF primers were: *primer 1* ; 5'-GCTCTTAGCAGACATTGGAAGA-3', complementary to nucleotides 783-804 and bFGF *primer 2* ; 5'-GGCTTCTTCCTGCGCATCCA-3' corresponding to nucleotides 451-470 of the human bFGF cDNA sequence reported by Kurokawa et

al. (1987). The corresponding sequences in rat bFGF are identical (Shimasaki et al., 1988).

β-actin *primer 1*; 5'-TGGCCTT^C/AGGGTTCAG^G/AGGGG-3' complementary to nucleotides 1613-1633 and β-actin *primer 2*; 5'-ATCGTGGG^G/cCGCCC^C/TAGGCA-3' corresponding to nucleotides 1256-1275 of the human β-actin genomic sequence (Genbank accession number X00351). Both primers differ from the corresponding rat actin sequence at 2 nucleotides, indicated by subscripts in the above sequences.

FGF receptor *primer 1*; 5'-GCCAGCAGTCCCGCATCATCA-3' , complementary to nucleotides 2180-2200 and *primer 2* ; 5'-GACGCAAC^A/$_G$GAGAA^A/$_G$GA^C/$_T$TTG-3' corresponding to nucleotides 1543-1563 of the genomic sequence reported for the transmembrane form of the receptor in human umbilical vein endothelial cells (Johnson et al., 1991). Subscripted letters in primer 2 denote differences between the human sequence and that reported for murine brain FGF receptor sequence (Reid et al., 1990) (Genbank accession number M28998). The predicted PCR products with these primer sets were: β-actin, 244 bp; bFGF, 352 bp; FGF receptor, 658 bp. All oligonucleotides were desalted on a Sephadex G-25 column and used in the PCR reactions without further purification.

IV. Molecular Biology Experiments

Brain and Pituitary Dissection

The brains were removed to ice cold Dulbecco's phosphate buffered saline (PBS; pH 7.4) for 1 min. to firm the tissue prior to dissection. The MBH was dissected with a razor blade via coronal cuts placed anterior to the mammillary bodies caudally, 2 mm posterior to the optic chiasm rostrally and to the lateral sulci laterally. The MBH fragment was removed at a depth of 2 mm using forceps

(MORIA forceps MC34B, Fine Science Tools, Vancouver). The mean weight of the MBH fragments was approximately 4 mg. The ME was gently removed in one piece using forceps. A fragment from the frontal cortex was obtained by two lateral cuts, one along the central sulci and one 3 mm lateral to the central sulci. Two coronal cuts were made approximately 5 and 7 mm rostral to Bregma. The block of cortex was removed at a depth of 2 mm using forceps. A portion of cerebellum of similar size was also removed. The posterior lobe of the pituitary was carefully separated from the anterior lobe under binocular magnification.

RNA Isolation and Reverse Transcription

Brain fragments were immediately transferred to individual 1.5 ml Eppendorf tubes and lysed in 0.5 ml guanidinium isothiocyanate containing 1% β-mercaptoethanol. RNA was isolated by sequential extraction with phenol and chloroform (see Puissant and Houdebine, 1990) and cDNA was prepared from total RNA (10 ng - 1 μg) using M-MLV reverse transcriptase and random hexanucleotide primers (pd(N)$_6$) (from Murphy et al., 1992). The RNA in each sample was quantified using a Beckman DU-600 spectrophotometer set at a wavelength of 260 nm. The concentration of RNA (ng/ml) is equal to Absorbance multiplied by 4000. Total RNA was placed in 10 μl of sterile water and heated at 90°C for 10 min., quick-chilled on ice (4°C) for 5 min. and then added to the reverse transcription (RT) reaction mixture in 1.8 ml microfuge tubes. Each reaction tube contained 5 μl of a 5-fold concentrated buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM MgCl$_2$), 100 mM of each of the four dNTPs, 0.04 nM pd(N)6, 0.01 M DTT, 40 U RNasin, 200 U MMLV reverse transcriptase and 5 mg RNA in a total volume of 25 μl. The reactions were incubated for 10 min. at 23°C followed by 3 hrs. at 42°C, and finally 7 min. at 95°C. They were then quick-

chilled on ice and stored at -20°C. One-fifth of the reverse transcription reaction was used for PCR amplification with each set of primers as described below.

PCR amplification

The PCR reaction mixtures (25 µl final volume) contained 100 µM of each of the four dNTPs, 0.75 U Taq polymerase, 2.5 µl of a 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0), 15 mM $MgCl_2$, 1% Triton X-100), 50 pmols of each primer, and 5 µl of the reverse transcription reaction. The reaction mixtures were overlayed with 100 µl light mineral oil and incubated in an Ericomp single block thermal cycler (Ericomp Inc., San Diego, CA). After an initial denaturation for 2 min. at 94 °C, the samples were amplified for 20-30 cycles, each consisting of annealing at 55 °C for 60 sec., elongation at 72 °C for 120 sec., and denaturation at 94 °C for 60 sec. The reaction products were visualized by ethidium bromide staining after electrophoretic separation on a 2% agarose gel at 60 V for 1 hr. The identity of the amplified products was confirmed by restriction enzyme analysis as previously described (Murphy et al., 1992). In all experiments samples containing no RNA were included as negative controls to monitor for contamination in the PCR reaction. In semi-quantitative experiments, [32P]-dCTP was included in the PCR reaction mix to permit autoradiographic detection and densitometry of the reaction products.

Northern Blotting

Total RNA was electrophoresed in 1% agarose-formaldehyde gels and transferred to nitrocellulose by capillary blotting or applied directly to nitrocellulose using a slot-blot apparatus (BRL-Life Technologies, Burlington, Ontario, Canada). After transfer to nitrocellulose, the blots were hybridized with [32P]-labeled bFGF cDNA overnight at 42°C in 6.6X sodium chloride phosphate buffer (SCP) containing 10% dextran sulfate (20X SCP consisted of 2M NaCl, 0.6

M Na_2HPO_4, and 0.02 M EDTA, pH 6.2). The blots were washed twice for 15 min. at 65ºC in 6.6X SCP/ 1% Sarcosyl, and for 3 hrs. at 65ºC in 1X SCP/ 1% Sarcosyl. The bFGF cDNA probe used was rat bFGF cDNA clone pRObFGF503, generously provided by Drs S. Shimasaki and A. Baird, Department of Molecular Endocrinology, The Whittier Institute, La Jolla, Ca. The β-actin probe was a 1.2 kb Bgl cDNA fragment corresponding to the coding region of the mouse β-actin cDNA. RNA expression was quantified by densitometric scanning after autoradiographic detection on Kodak X-Omat AR X-ray film at -70 ºC. For each sample, bFGF cDNA was divided by the quantity of actin cDNA as an internal control.

<u>Slot Blotting</u>

In some experiments the samples were transferred directly to nitrocellulose using a slot blot technique. The amplified DNA samples were placed in individual slots of the Schleicher and Schuell minifold II. A vacuum pump was applied to the apparatus and the samples were rapidly filtered, leaving the DNA on the nitrocellulose paper. Each sample was washed 5 times with 25 ml of 10x SSC to assure that nonspecific radioactivity was washed through the system. The DNA was crosslinked to the nitrocellulose paper using a Stratagene UV Stratalinker 2400. X-ray film was placed over the nitrocellulose and incubated at -70 ºC for 30 min. and 18 hrs. The nitrocellulose underlying each slot was excised and the associated radioactivity was measured by scintillation counting.

V. Analysis of bFGF-li

<u>Immunocytochemistry for bFGF-li</u>

The procedure used in the detection of bFGF-li is almost identical to that described for FLI in Chapter 3 (Material and Methods section). The following changes to our standard protocol were used for bFGF-li:

(i) Tissue sections were incubated in 2% normal horse serum (NHS; Gibco Laboratories, NY; 200 6050AG) and 0.5% bovine serum albumin (BSA; Sigma, MO; A-4503) in PBS and TX for 1 hr. to block non-specific binding by the primary (1°) antibody.

(ii) After the blocking step, the sections were incubated for 5 days at 4°C with a mouse monoclonal bFGF antibody (UBI, New York; Cat.# 05-118; see Appendix C for spec sheet) at a concentration of 1.8 mg/ml in 2% BSA.

(iii) After the incubation, the sections were washed three times (10 min. per wash) in PBS and TX and incubated in rat-adsorbed biotinylated horse anti-mouse antibody (Vector Laboratories; BA 2001) at a dilution of 1:200 in PBS at room temperature for 1 hr.

(iv) After three washes, the sections were incubated with avidin-biotin-peroxidase complex (Elite ABC kits; Vector Laboratories; PK-6100) at a dilution of 1:200 (5 ml each of solution A and B/ml) in PBS at room temperature for 1 hr.

Densitometric Analysis of Immunocytochemistry

Composite colour images of microscope slides were transmitted to a Macintosh Quadra 660AV computer using a Javelin model JE3662RGB video camera mounted on a Leitz Laborlux-S microscope. Images were captured using *Video Monitor* software (Apple Computers Inc., USA) and converted to greyscale Tag Image File Format (TIFF) files using NIH Image software (version 1.55b57, National Institute of Health, USA). Uniform brightness and contrast was achieved by adjusting the overall level of each image using Adobe Photoshop (version 2.5, Adobe, USA). The program was set to measure objects within a specific size range (5 to 160 pixels) and above a defined density or threshold where black (maximum) is equivalent to a numerical value of 250. The threshold was set at 200 and 150 for experiments 7 and 8, respectively. The number of

bFGF-positive cells within one half of the ARCN within the set criteria were counted using NIH Image software. The mean cell count per unit area was calculated for each animal from the eight coronal tissue sections through the ARCN.

VII. Experiments

(1) Optimization of PCR conditions for semi-quantitative analysis

In order to characterize the kinetics of the PCR reaction, serially diluted samples of total RNA (10 ng - 1 μg) from a human glioma cell line known to express high steady-state levels of bFGF mRNA (Murphy et al., 1990) were reverse transcribed and subjected to PCR amplification in the presence of bFGF oligonucleotide primers and [^{32}P]-dCTP (50,000 cpm/μl). Aliquots of the reactions were withdrawn at regular intervals from 10 to 40 cycles and the reaction products were separated by agarose gel electrophoresis as described above. The gels were transferred to nitrocellulose membranes by capillary blotting (Maniatis et al., 1982) and the DNA was immobilized by UV crosslinking. The reaction products were visualized by autoradiography (Xomat-AR, Kodak) at -70 °C for 30 min. to 18 hrs. and quantified by densitometric scanning. The radioactive bands were then excised and the associated radioactivity was measured by scintillation counting. Values determined by densitometry were linearly related to associated radioactivity over a broad range from 150 to 70,000 DPM (R^2=0.986, data not shown).

Individual sample values for bFGF were standardized against the level of actin expression in each sample. Actin is often used as a control gene because its expression is linearly related to the total amount of RNA, thereby reflecting the size of the tissue sample or the efficiency of the RT-PCR reaction.

The second part of this experiment was designed to establish the appropriate PCR conditions for amplification of the three sequences of interest from rat brain samples. Total RNA was isolated from the MBH of untreated female rats (P28; n=3) and 100 ng was reverse transcribed as above. Aliquots representing one-fifth of the reverse transcription reaction were withdrawn and subjected to PCR amplification in the presence of primers specifying bFGF, bFGF receptor and β-actin. Levels of bFGF and FGF receptor product were standardized against β-actin levels amplified from the same reverse transcription reaction. Samples in which β-actin failed to amplify were excluded from analysis. A negative control sample was amplified for 40 cycles without input cDNA.

(2) Quantitation of bFGF and bFGF.R mRNA in CNS by RT-PCR

Neural tissue from P34 female rats (n=3; VO; Mean Wt: 116.7 ± 6.1 g) was examined for FGF and FGF receptor mRNA. The brains were dissected as described above and included the following structures: MBH, cortex (CTX), cerebellum (CRB), anterior pituitary (AP), posterior pituitary (PP) and ME. The tissue from each structure was pooled from the 3 rats and the RNA was extracted, reverse transcribed and amplified as described above. As a negative control for RNA contamination, sterile water was also reverse transcribed and amplified using PCR. The experiment was repeated at a later date because we believe the first ME sample was degraded.

(3) Quantification of bFGF and bFGF.R mRNA Following NMDA Treatment

Animals were injected daily from P24 to P28 with NMDA (20 mg/kg in 0.1 ml saline, s.c.; n=9) or with saline only (n=5). Body weights were measured between 0900 h and 1000 h and injections were administered between 1100 h and 1200 h each day. At 29 days of age the rats were weighed and checked for

vaginal opening. Based on pubertal status the NMDA-treated group was divided into two subgroups (VO and nonVO). The rats were sacrificed by decapitation and the brains prepared for microdissection. The MBH and a sample of frontal cortex was dissected and prepared for RNA extraction, reverse transcription and amplification as described above. The uterus was removed, trimmed of fat and weighed. The oviducts were checked microscopically for the presence of eggs and the number of corpora lutea (CL) in the ovaries were counted.

(4) Quantification of bFGF and bFGF.R mRNA Following PMSG Treatment

At 1600 h on P26, female rats (n=9 per group) were injected s.c. with PMSG (20 IU in 0.1ml saline) or with vehicle only. On P29 between 1000 h and 1100 h the rats from both groups (PMSG and SAL) were weighed, checked for vaginal opening (VO) and then sacrificed by decapitation. The brain was rapidly removed and placed in ice-cold DPBS (pH 7.4) for 1 minute. The medial basal hypothalamus (MBH) and cerebral cortex were microdissected and processed individually for RNA isolation as described above. The reproductive organs were examined as described in expt. 3.

(5) bFGF-li in the ARCN in the NMDA Model of Puberty

Animals were injected daily from P24 to P28 inclusive with NMDA (20 mg/kg in 0.1 ml saline, s.c.; n=10) or with saline only (n=8). Body weights were measured between 0900 h and 1000 h and injections were administered between 1100 h and 1200 h each day. Twenty-four hrs. after the last injection (P29) the rats were weighed and checked for vaginal opening (VO). Rats from the NMDA-treated group that had reached VO and an equal number of rats from the saline-treated group (nonVO) (n=4/group) were anaesthetized, perfused and brains removed and sectioned as described previously. At 1100 h on P33, rats from the NMDA- and saline-treated group (n=4/group; all at VO) were anaesthetized,

perfused and brains removed and sectioned. Eight coronal tissue sections (50 μm) through the arcuate nucleus of each animal were stained for bFGF-li. The number of bFGF-li cells in the ARCN were analyzed and quantified with an image analysis system as described above.

(6) bFGF-li in the ARCN in the PMSG Model of Puberty

At 1600 h on P26 rats were injected (s.c.) with PMSG (20 IU in 0.1ml saline) or saline vehicle (0.1 ml; s.c.) (n=12/group). At 1600 h on P27, P28 and P29 the rats were weighed and checked for vaginal opening (VO). On each day, 4 rats from the experimental and control group were anaesthetized, perfused and brains removed for sectioning. In the PMSG treated group P27 to P29 corresponds to preproestrus, proestrus and estrus (VO), respectively. Eight coronal tissue sections (50 μm) through the arcuate nucleus of each animal were stained for bFGF-li. The number of bFGF-li cells in the ARCN were analyzed and quantified as described above.

VIII. Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA) using Abacus Concepts, Statview 4.0 program for Macintosh computers. Significant differences between groups were determined by *post hoc* tests such as Bonferroni/Dunn. Statistical significance was inferred at $p < 0.05$.

Results

(1) Optimization of PCR conditions for semi-quantitative analysis

As shown in Fig. 48B, the RT-PCR method can readily amplify a specific DNA sequence from less than 10 ng of total RNA and differences in mRNA abundance of less than 3-fold were reliably detected. Since only one-fifth of the reverse transcription reaction was used in the PCR amplification, this represents less than 2 ng of cDNA, assuming 50% efficiency of the M-MLV reverse transcriptase (Rappolee et al., 1989). The limits of resolution were strongly dependent on both the starting concentration of RNA and on the number of cycles of amplification (Fig. 48A). Resolution declined dramatically when the plateau phase of the reaction was reached, in this case between 25 and 40 cycles of amplification. However, as demonstrated in Fig. 48B, when the amplifications were performed at limiting cycle number, differences in starting RNA concentration of less than 3-fold were readily discernible over a wide range of input RNA concentrations spanning two orders of magnitude.

This experiment was designed to establish the appropriate PCR conditions for amplification of the three sequences of interest from rat brain samples. Total RNA was isolated from the medial basal hypothalamus (MBH) of untreated rats and 100 ng was reverse transcribed and subjected to PCR amplification in the presence of primers specifying bFGF, bFGF receptor and β-actin. As shown in Fig. 49, amplification of the bFGF and bFGF receptor products remained in the linear (exponential) portion of the curve between 15 and 30 cycles, after which accumulation of the product began to plateau. Under identical reaction conditions the amplification curve for β-actin was shifted to the left by approximately 5 cycles and reached the plateau phase after 25 cycles, reflecting the greater abundance of β-actin mRNA relative to the other two transcripts. The

product sizes (in bp) are β-actin, 244; bFGF, 352; FGF receptor, 658 which are consistent with the predicted products for these sets of primers. The identity of the amplified products was further confirmed by diagnostic restriction enzyme analysis and by high stringency hybridization of the amplified products with the corresponding full-length cDNAs (data not shown).

Figure 48. - Characterization of the bFGF RT-PCR reaction.

(a) Quantitation of differences in input bFGF mRNA as a function of PCR cycle number. Total RNA from human U87-MG glioma cells was diluted to the indicated concentrations prior to reverse transcription and PCR with [^{32}P]-dCTP and bFGF-specific oligonucleotide primers as described in Materials and Methods. Aliquots of each reaction were removed after 10, 20, 30 or 40 cycles and the reaction products were subjected to agarose gel electrophoresis, autoradiography and liquid scintillation counting. Total RNA used in the reverse transcription reactions were 10 (□), 30 (◇), 100 (●), 300 (△) or 1000 (■) ng. One fifth of each reaction was employed in the PCR amplification.

(b) Effect of input RNA concentration on PCR product yield. The values plotted here are derived from the 20 cycle amplifications depicted in panel A.

A

B
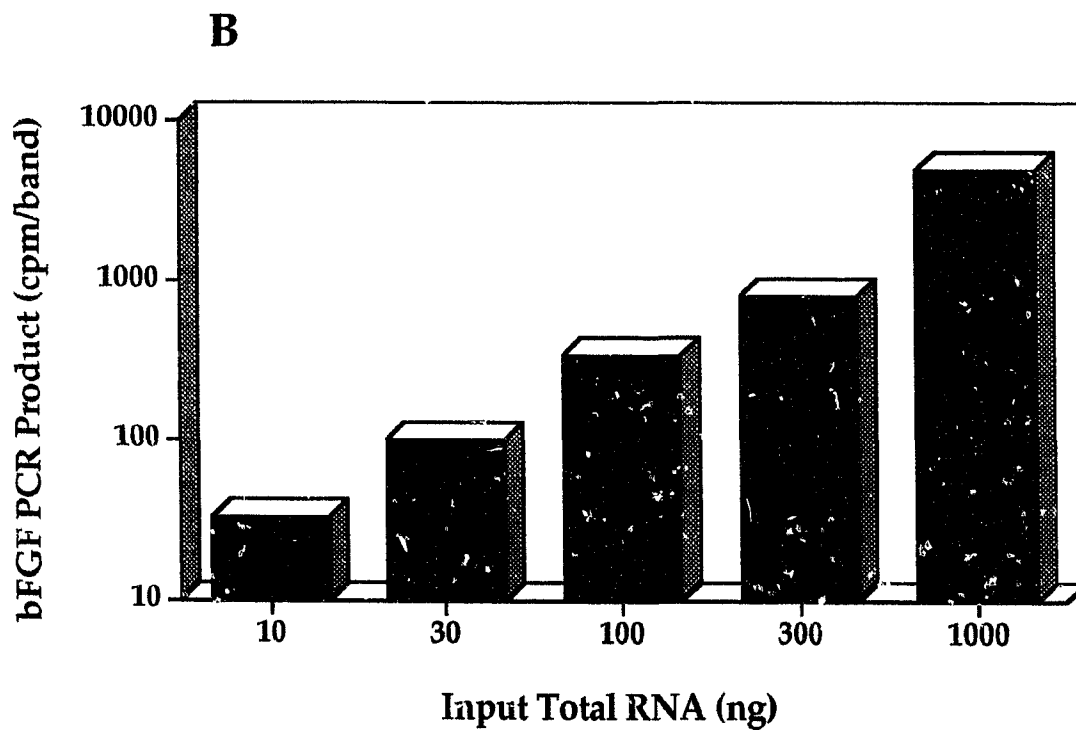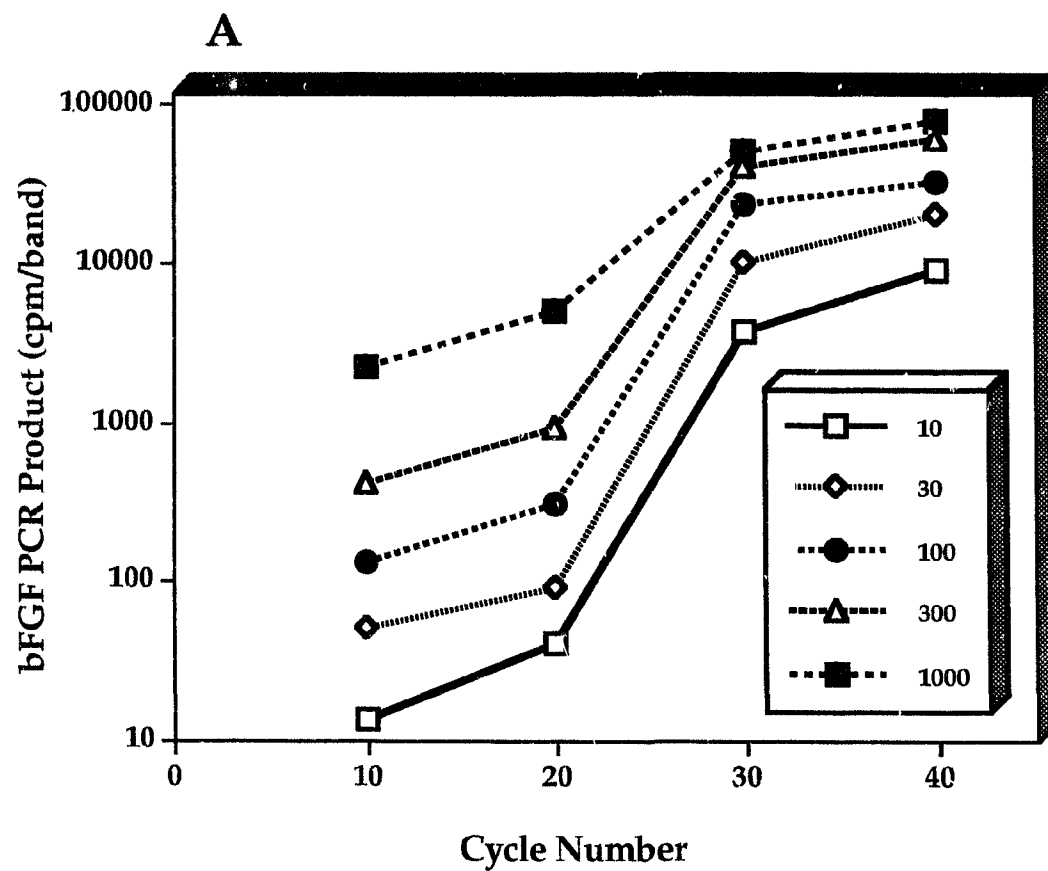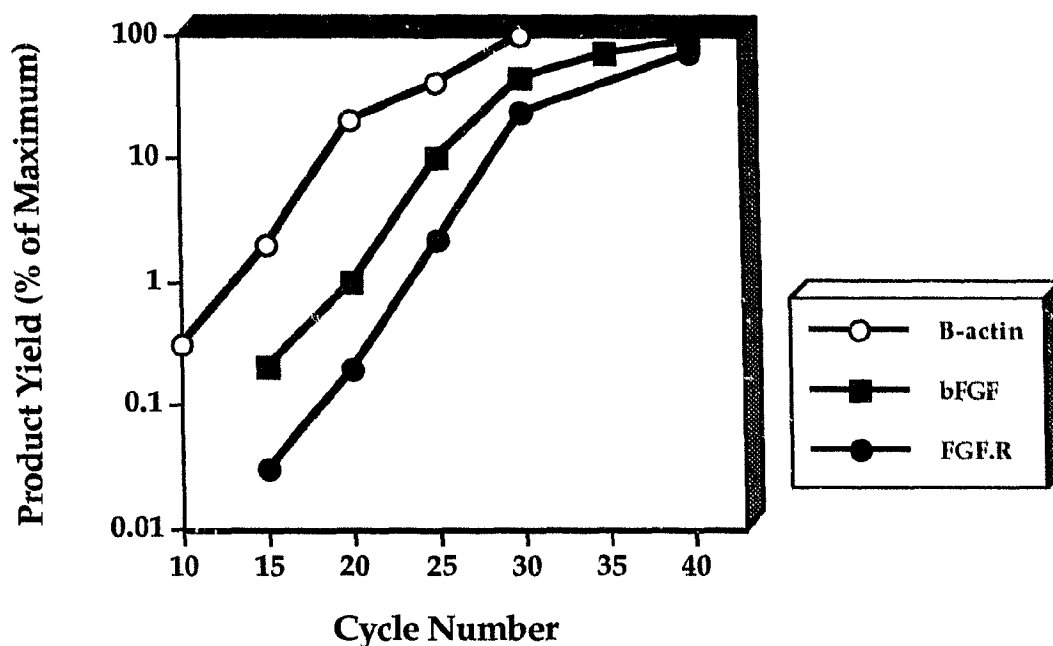
Figure 49. - Optimization of PCR conditions for semi-quantitative analysis.

(a) Total RNA (100 ng) from rat hypothalamus was reverse transcribed and subjected to PCR amplification in the presence of [^{32}P]-dCTP and specific primers for β-actin (○), FGF receptor (●) and bFGF (■). Aliquots were withdrawn after the indicated number of cycles and subjected to agarose gel electrophoresis, autoradiography and liquid scintillation counting. Product yield was calculated as the ratio ([^{32}P] cpm specifically incorporated/ [^{32}P] cpm added). Values are expressed as a percentage of the maximum product yield for each reaction.

(2) Quantitation of bFGF and bFGF.R mRNA in the CNS by RT-PCR

PCR amplification of reverse-transcribed RNA from rat brain tissue and pituitary generated the predicted PCR products for FGF receptor , bFGF, and β-actin (Fig. 50). The FGF receptor product was strongly amplified after 25 cycles in samples from several brain regions as well as anterior and posterior pituitary. In contrast, although the bFGF PCR product was strongly amplified in cortex, cerebellum and medial basal hypothalamus, it was only faintly detectable in the anterior and posterior pituitary after 25 cycles. However, after 30 -40 cycles of amplification the bFGF product was readily detectable in both pituitary lobes, indicating that bFGF mRNA is present at low levels in the pituitary, possibly in a small subpopulation of pituitary cells or in associated vascular endothelial cells . The relative level of expression of bFGF was MBH > cerebral cortex = cerebellum >> pituitary. The apparent low levels of bFGF mRNA in the pituitary are in accord with a recent report by Gonzalez et al. (1992) that bFGF mRNA is not detectable in either lobe of the pituitary by *in situ* hybridization.

The median eminence (ME) RNA sample shown in this experiment was probably degraded, and no product was amplified with any of the primers tested, including β-actin. This experiment was repeated and both FGF and FGF.R mRNA were localized to the ME. The relative expression of bFGF and bFGF receptor mRNA in these tissues, after standardization against b-actin, are shown in Fig. 51.

Figure 50. - Detection of bFGF and bFGF receptor expression in rat tissues by RT-PCR.

The products shown in the autoradiogram are the result of 20 cycles (β-actin) or 25 cycles (bFGF and FGF receptor) of amplification. 100 ng of total RNA was reverse transcribed and subjected to PCR amplification in the presence of [^{32}P]-dCTP and specific primers for FGF receptor (FGF-R), bFGF product or β-actin. MBH, medial basal hypothalamus; ME, median eminence; AP, anterior pituitary; PP, posterior pituitary; Co, cortex; Ce, cerebellum.

MBH ME AP PP Co Ce

FGF-R

FGF

Actin
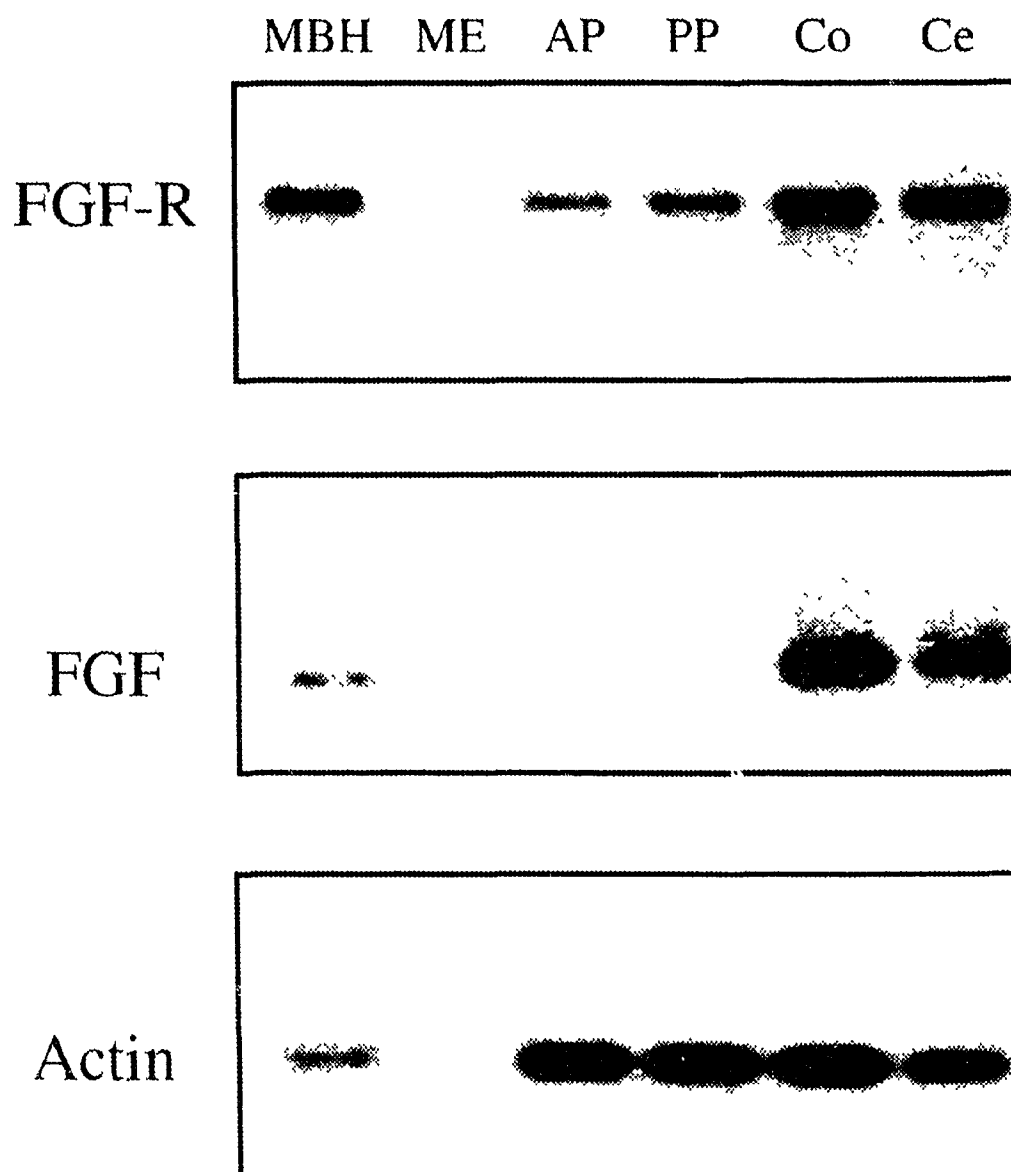
Fig. 50
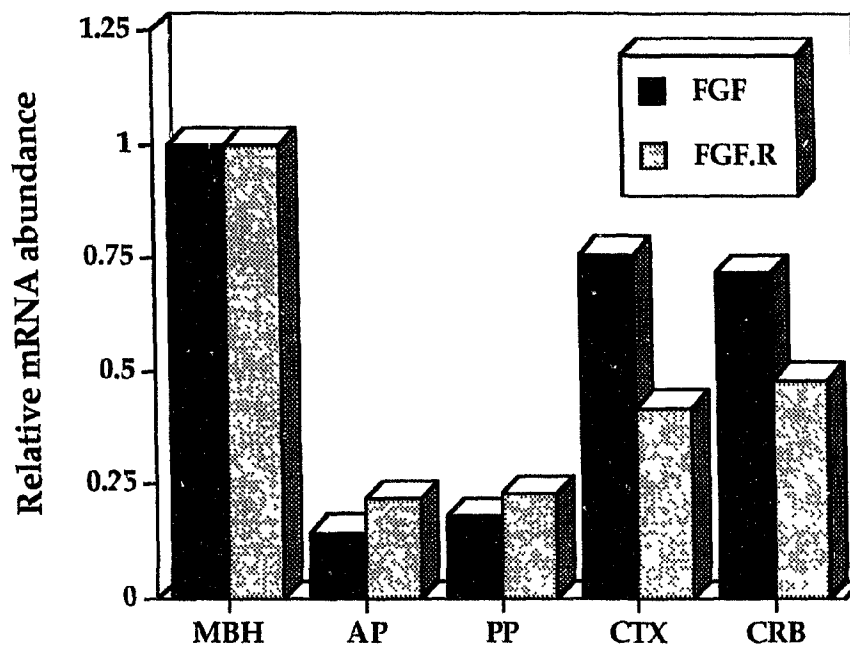
Figure 51 - Relative abundance of bFGF and bFGF.R mRNA in different areas of the rat brain.

The relative abundance of bFGF (solid bars) and FGF-R (dotted bars) mRNA was estimated by densitometric scanning of the autoradiograms shown in figure 50. The data were standardized against the level of β-actin product. Values are expressed relative to the level of expression in MBH, which is arbitrarily set at 1.

(3) Quantification of bFGF and bFGF.R mRNA Following NMDA Treatment

We have previously reported that single daily injections of the glutamate agonist NMDA accelerates sexual maturation in female rats, indicating a role for hypothalamic glutamate receptors in the regulation of sexual maturation (MacDonald and Wilkinson, 1990; see also Chapter 2). We investigated the effect of NMDA treatment on bFGF expression. As summarized in Table 4, of 9 animals injected daily from 24 to 28 days of age with NMDA, 5 achieved first ovulation by P28 as defined by vaginal opening, and presence of corpora lutea. In contrast, none of the saline-injected controls reached first ovulation during the course of the experiment.

NMDA-treated animals were sub-divided into 2 groups based on their status as VO or nonVO, and sacrificed for RNA isolation and PCR amplification. As shown in Figure 52, NMDA treatment caused a decline in bFGF PCR product abundance in the MBH of NMDA-treated VO animals compared to NMDA - or saline-treated non-VO animals. The results of this experiment are summarized in Fig. 53. Expression of bFGF mRNA in the medial-basal hypothalamus of the NMDA-treated VO group was significantly ($p<0.05$) reduced by 50% vs. saline-treated controls (Fig. 53A). In contrast, the expression of bFGF mRNA in the cortex of the NMDA-treated VO group was not significantly different from saline-treated controls (Fig. 53B). Expression of bFGF mRNA in the NMDA-treated animals that did not reach VO was intermediate between control and VO levels and was not significantly different from controls. The expression of FGF receptor in hypothalamus and cortex was not significantly altered by NMDA treatment. The experiment described here was repeated twice with similar results.

Table 4. - Effect of NMDA treatment on first ovulation, body weight, uterine weight and presence of ova and corpora lutea (CL) on P29. The number of rats at VO were separated into those that had ovulated and those that were in proestrous (ProE).

Values are mean ± s.e.m.

	Control	NMDA (nonVO)	NMDA (VO)
n	4	4	5
Body Wt (g)	91.0 ± 3.9	87.8 ± 2.7	92.2 ± 3.7
Uterine Wt (mg)	86.4 ± 5.1	133.1 ± 17.9	145.8 ± 6.8
# Rats at VO	0/4	0/4	5/5
#Rats at ProE	0/4	1/4	0/5
#Rats with ova and CL	0/0	0/0	5/5
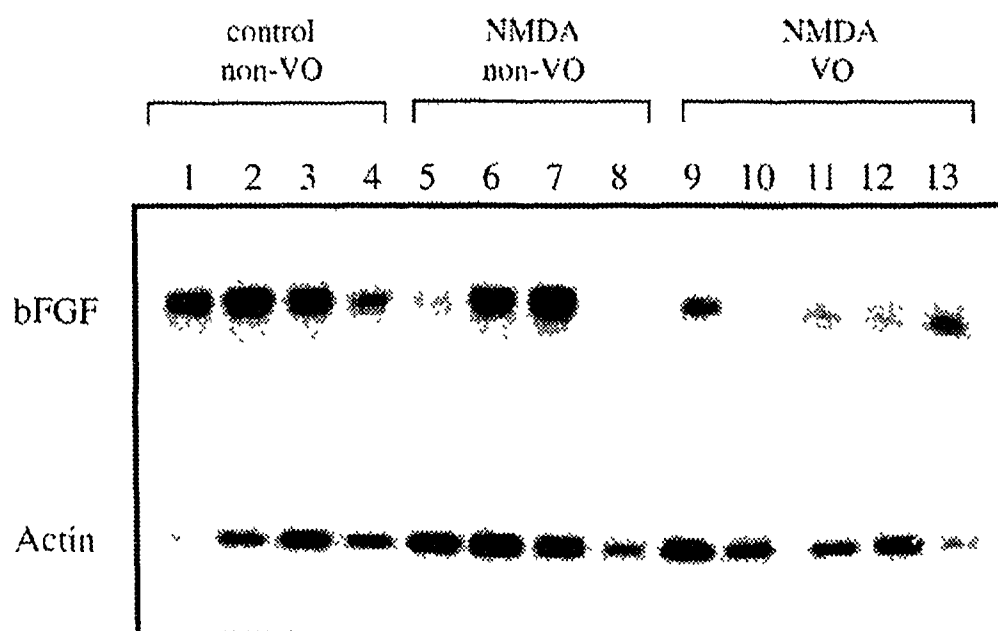
Figure 52. - Autoradiographic detection of bFGF and actin PCR products from MBH of control and NMDA treated rats.

Rats received 5 daily injections of NMDA (20 mg/kg) or saline vehicle (control) from P24 to 28. Maturational status (vaginally opened (VO) or non-vaginally opened (NonVO)) was determined at sacrifice on P29. RNA was isolated from individual rats and subjected to reverse transcription and PCR amplification in the presence of $[^{32}P]$-dCTP and specific primers for bFGF and β-actin.

Figure 53. - Effect of NMDA treatment on expression of bFGF and FGF receptor mRNA in the hypothalamus and cortex.

Rats received 5 daily injections of NMDA (20 mg/kg) or saline vehicle (control) from P24 to 28. On P29 the NMDA-treated animals were assigned to sub-groups based on vaginal status (nonVO, no vaginal opening; VO, vaginal opening). All of the control animals were nonVO. RNA was isolated and the relative abundance of bFGF (black bars) and FGF receptor (dotted bars) mRNAs was determined as described in the legend to Fig. 52. (A) hypothalamus; (B) cortex.
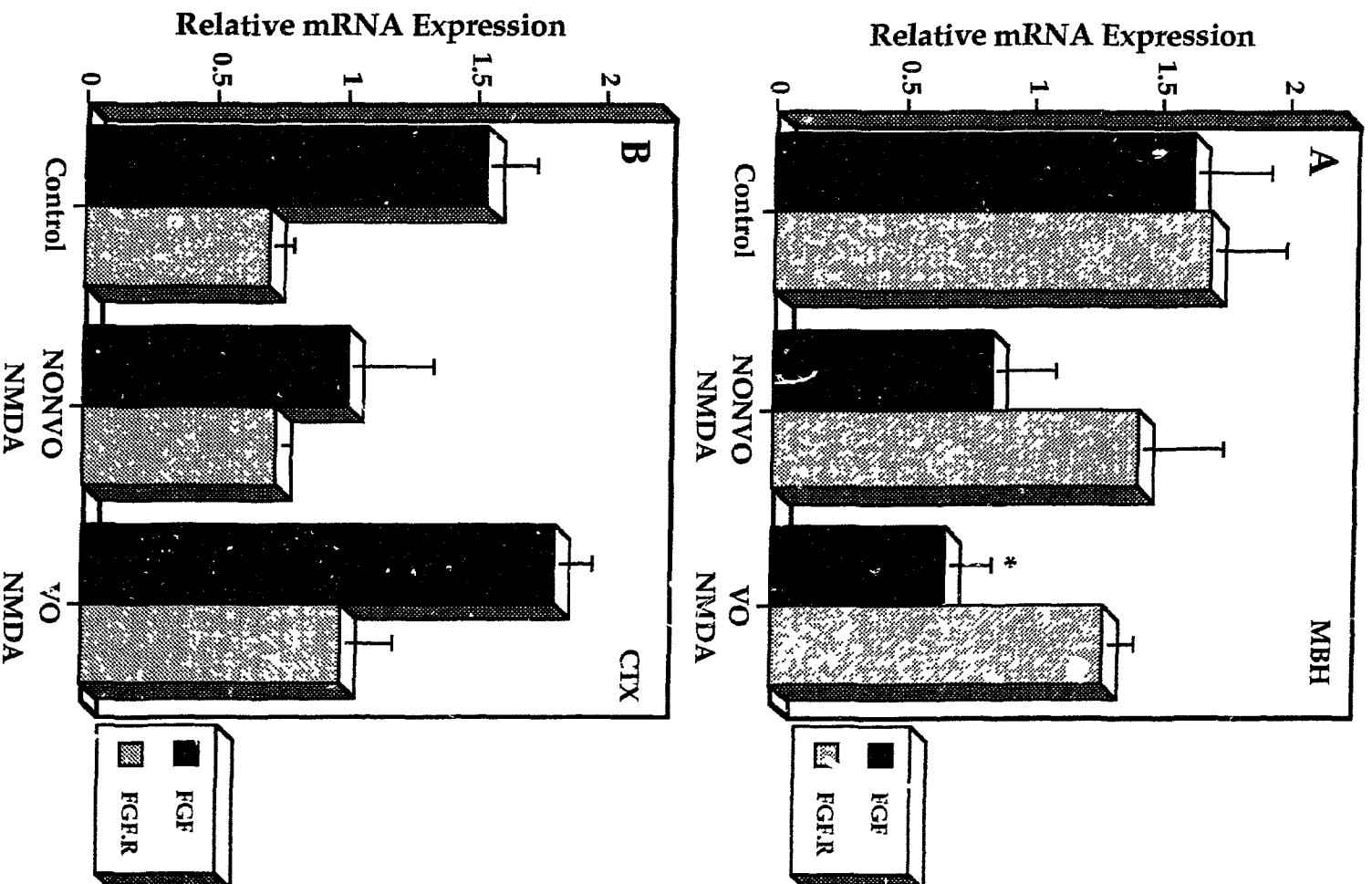
(4) Quantification of bFGF and bFGF.R mRNA Following PMSG Treatment

It was necessary to determine whether the decline in hypothalamic bFGF mRNA levels was related to pubertal changes in hypothalamic function or a specific effect of NMDA on glutamate receptors. Therefore, we chose to induce first ovulation by injection of pregnant mare's serum gonadotropin (PMSG). Treatment of prepubertal female rats with PMSG on the afternoon of P26 resulted in 100 % of the animals achieving first ovulation on P29 (Table 5). The saline-treated animals did not reach first ovulation on P29. RNA was isolated from MBH and cortex of individual animals and subjected to RT-PCR. As shown in Fig. 54A, PMSG treatment significantly reduced bFGF mRNA abundance in the MBH by 40% relative to saline treated control animals ($F_{1,13}$=11.461; p = 0.0049) but had no effect on bFGF expression in cortex from the same animals ($F_{1,13}$= 0.013; P = 0.9105) (Fig. 54B). There was no significant effect of PMSG on bFGF receptor expression in MBH or cortex.

To confirm the RT-PCR results, the PMSG experiment was repeated as described above except tissue samples from 10 rats per group were pooled to provide enough RNA for slot-blot analysis. The effect of PMSG on uterine weight and ovulatory status was similar to the first experiment (Table 6). Serial dilutions of total RNA (25 - 1.5 µg/slot) from MBH and cortex of saline or PMSG-treated rats were applied to nitrocellulose and probed sequentially with [^{32}P]-labeled cDNA probes for rat bFGF and mouse β-actin. Northern hybridization with the bFGF cDNA probe confirmed the presence of a single 6 kb mRNA transcript in rat hypothalamus. The results obtained by slot-blot hybridization confirm the RT-PCR findings (Fig. 55). The relative level of bFGF expression (estimated from the initial slopes of the dilution series) was reduced in PMSG-treated MBH by 35-40% relative to saline treated controls. bFGF expression in cortex was unaffected by PMSG treatment.

Table 5. - Effect of PMSG treatment on first ovulation, body weight, uterine weight and presence of ova and corpora lutea on P29 (#1). The number of rats at VO were separated into those that had ovulated and those that were in proestrous (ProE).

Values are mean ± s.e.m.

	Control	PMSG
n	9	9
Body Wt. (g)	88.7 ± 1.2	88.1 ± 1.5
Uterine Wt. (mg)	94.2 ± 16.1	130.3 ± 5.6
# Rats at VO	0/9	7/9
# Rats in ProE	2/9	0/9
#Rats with CL	0/9	9/9

Figure 54. - Effect of PMSG treatment on expression of bFGF and FGF receptor mRNA in hypothalamus and cortex.

RNA was isolated from individual rats 3 days after a single injection of saline (control) or PMSG and subjected to reverse transcription and PCR amplification in the presence of primers for bFGF, FGF receptor or β-actin. Basic FGF (solid bars) and FGF receptor (dotted bars) were standardized against the level of β-actin, and are expressed as a percentage of the corresponding control value.

Relative mRNA Expression

Relative mRNA Expression

A

B

MBH

CTX

Control

PMSG

Control

PMSG

0 20 40 60 80 100 120

0 20 40 60 80 100 120

*

FGF
FGF.R

FGF
FGF.R
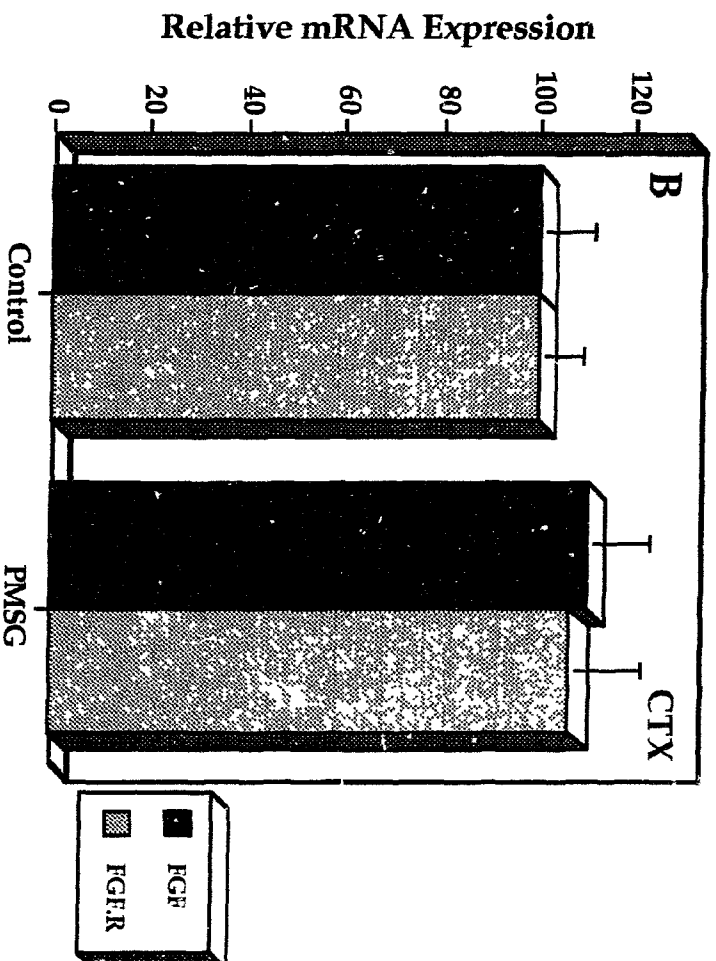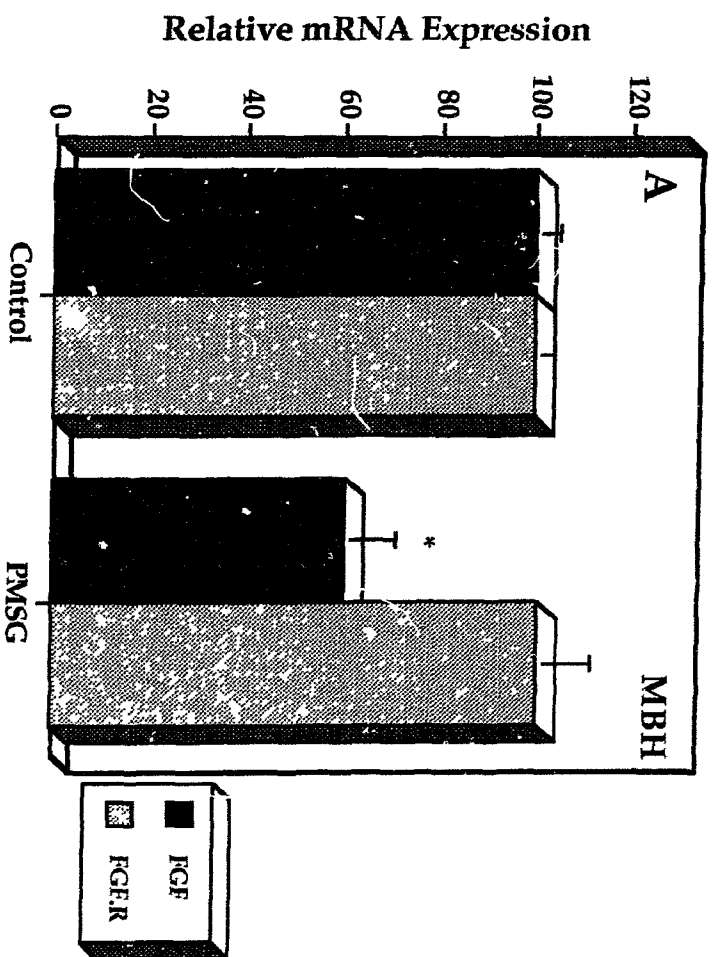
Table 6 - Effect of PMSG treatment on first ovulation body weight, uterine weight and presence of ova and corpora lutea on P29 #2). The number of rats at VO were separated into those that had ovulated and those that were in proestrous (ProE).

Values are mean ± s.e.m.

	Control	PMSG
n	10	11
Body Wt. (g)	86.4 ± 1.2	82.9 ± 1.1
Uterine Wt. (mg)	77.9 ± 8.3	139.8 ± 6.1
# Rats at VO	0/9	9/9
# Rats in ProE	0/9	0/9
#Rats with CL	0/9	9/9
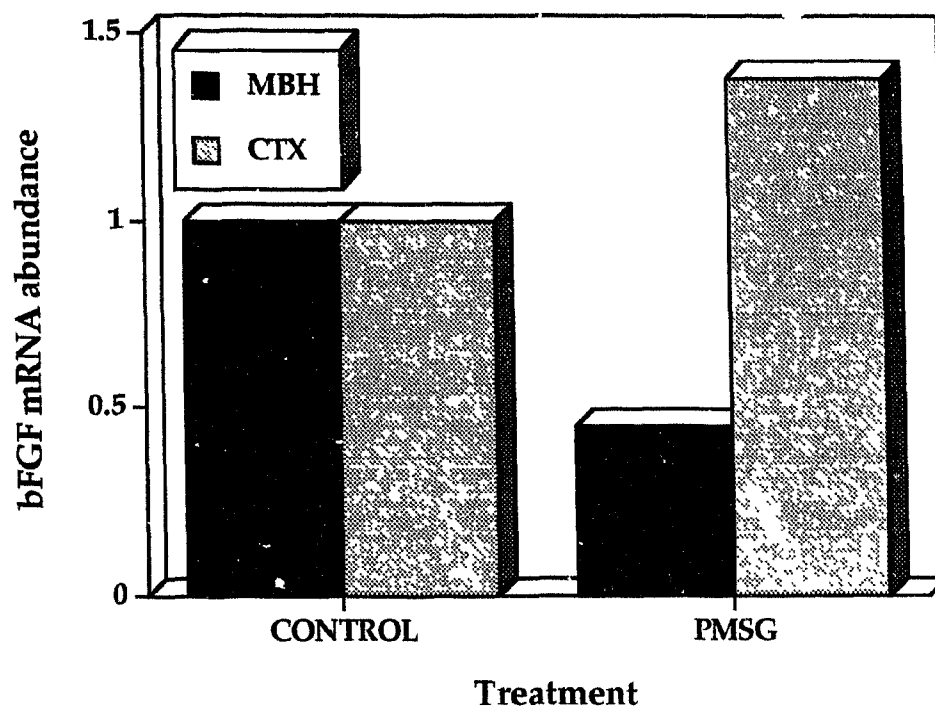
Figure 55 -. RNA slot-blot estimation of bFGF mRNA expression in hypothalamus and cortex in response to PMSG treatment.

Total RNA pooled from 10 rats was serially diluted and applied to nitrocellulose using a slot blot apparatus. The blots were probed sequentially with [^{32}P]-dCTP labeled cDNA probes for bFGF and β-actin. The relative levels of bFGF were standardized against the level of β-actin in each sample.

(5) bFGF-li in the ARCN in the NMDA Model of Puberty

Juvenile female rats were treated with NMDA or vehicle as per our standard model of NMDA induced puberty and examined on P29 and P33 for bFGF-li in the MBH. Small (<10 μm) circular or oval bodies were clearly labeled and regionally localized throughout the brain. The immuno-reactivity appeared to be both nuclear and cytoplasmic but processes were not visible and the possibility remains that these "small cells" may be nuclei. Occasionally, a dark circular structure within the cell was visible possibly representing the nucleus or nucleolus. Very dense staining was observed in the cells lining the ventricles and in cells within the meningial layers surrounding the section.

As illustrated in Figure 56, the P29 NMDA-treated rats (VO; Fig. 56A) exhibited more cellular staining in the ARCN than age-matched controls (nonVO; Fig. 56B). There are many more densely labeled bFGF-positive cells in the ventrolateral portion of the ARCN of the NMDA group compared to controls (see arrows). In contrast to this, there is less bFGF-li in the ARCN in the NMDA-treated rats compared to controls on P33 (Fig. 56C and D; see arrows). Our subjective evaluations of the brain sections were confirmed by densitometric analysis. Fig. 57 illustrates the mean number of bFGF-positive cells per unit area for treated and control groups on P29 and P33. A 2 factor ANOVA revealed significant effects of treatment ($F_{1,11}$=5.825; p=0.0327), age ($F_{1,110}$=44.942; p<0.0001) and a treatment by age interaction ($F_{1,11}$=55.573; p<0.0001). *Post hoc* Bonferroni/Dunn tests indicated that on P29, there were significantly (p<0.001) more bFGF-positive cells/unit area in NMDA vs. control groups. In contrast, significantly (p<0.05) fewer bFGF-positive cells were observed in NMDA-treated vs. controls on P33.

Figure 56. - bFGF-li in the ARCN of juvenile female rats treated with NMDA or saline for 5 days.

Photomicrograph of bFGF-li in the MBH of female rats on P29 and P33 treated from P24 to P28 with NMDA (20 mg/kg) or saline (0.1 ml). On P29, NMDA-treated (A) has markedly more densely labeled bFGF-positive cells in the ARCN compared to age matched controls (B). Interestingly, the strong staining observed on P29 in NMDA-treated group is absent on P33 (C) and there appears to be more bFGF-positive cells in the control group (D) Scale bar = 100 μm.

NMDA (P29)   Control (P29)

NMDA (P33)   Control (P33)
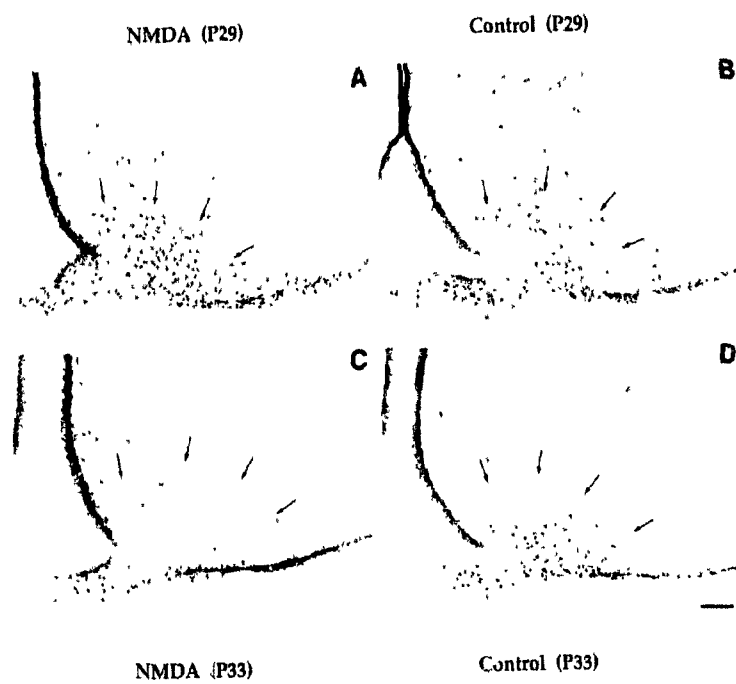
Fig. 56
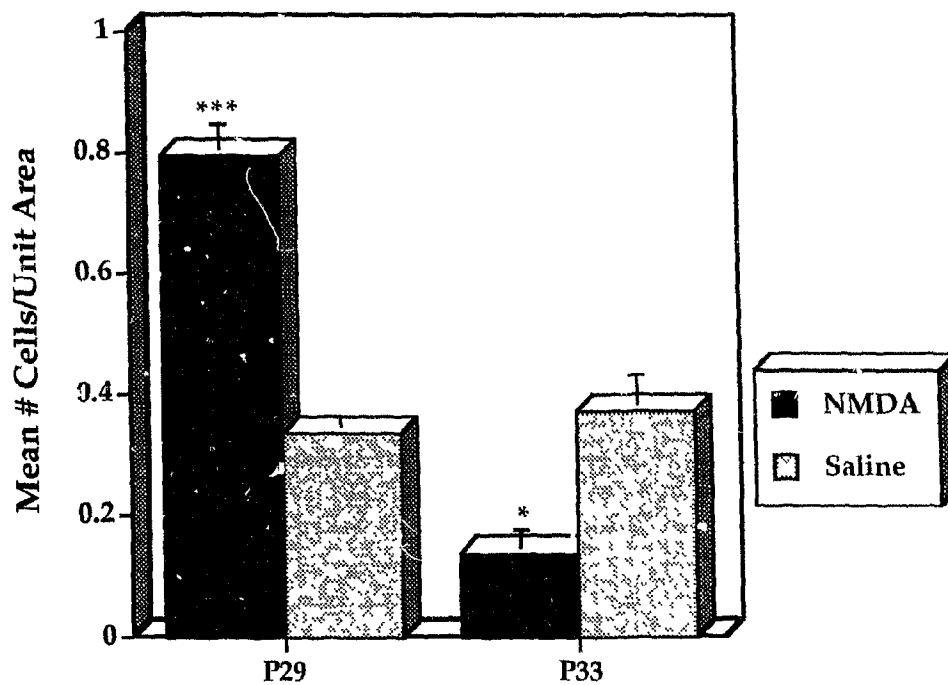
Figure 57. - The mean number of bFGF-li cells in the ARCN of NMDA- or saline-treated juvenile female rats.

Bar graph showing the mean number of bFGF-li cells in the ARCN of NMDA- or saline-treated rats at two time points. Female SD rats were injected (s.c.) at 1100 h each day with NMDA (20 mg/kg) or saline (0.1 ml) from P24 to P28 Animals from each group were sacrificed and perfused on the morning of P29 and P33 (n=4/group). Densitometric analysis of bFGF-li in one half of the ARCN revealed significantly (***$p<0.001$) more bFGF-positive cells in the NMDA-treated group at P29 and falling significantly (*$p<0.05$) below the control group by P33. Values shown are mean ± sem.

(6) bFGF-li in the ARCN in the PMSG Model of Puberty

As in the PCR experiments, we wanted to analyze whether levels of bFGF expression in the ARCN changed if puberty is stimulated via a non-excitotoxic means (i.e. PMSG). The PCR experiments showed a similar trend in bFGF mRNA expression in both PMSG and NMDA treated experiments compared to controls. However, in this experiment there does not appear to be any difference in bFGF-li in the ARCN of PMSG vs. saline-treated female rats at any time point during the experiment (Figure 58). The 2 factor ANOVA revealed a significant effect of time ($F_{2,18} = 3.607$, p=0.0482) but not treatment ($F_{1,18} = 0.311$, p=0.5837) on the mean cell count per unit area. *Post hoc* Bonferroni/Dunn tests indicate that treatment and control groups are not significantly different at any time point.
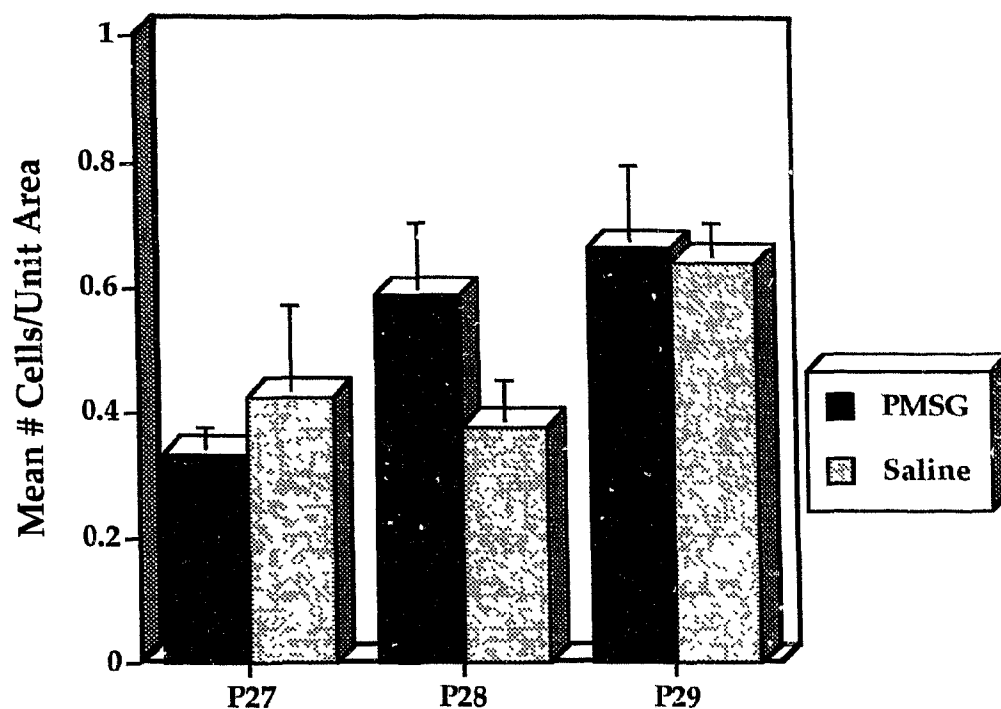
Figure 58 - The mean number of bFGF-li cells in the ARCN of PMSG- or saline-treated juvenile female rats.

Results of densitometric analysis of bFGF-li in the ARCN of female rats at various ages (P27, P28, P29) treated with saline or PMSG (20 IU) on P26 (n=4/group/time point). The mean number of bFGF-positive cells per unit area in one half of the ARCN is plotted for each treatment group at different ages. For the PMSG group, the days correspond to preproestrus (P27), proestrus (P28) and estrus (P29; VO). In both groups there appears to be an increasing trend in the number of bFGF-positive cells over time. However, the treatment and control groups are not significantly different from one another at any age. Values are mean ± sem.

Discussion

The study of bFGF expression *in vivo* has previously been impeded by the low copy numbers and short half-life of the mRNA. The development of the PCR technique (Saiki et al., 1988) and its implementation in conjunction with reverse transcription of RNA (Rappolee et al., 1988b) has made it possible to detect and semi-quantitate multiple low abundance mRNA species in individual small samples (Rappolee et al., 1988a). Using $[^{32}P]$-dCTP in the master mix, the sensitivity of this technique is estimated to be four orders of magnitude more sensitive than northern hybridization (Hoof et al., 1991). Although some earlier studies indicated unacceptable tube-to-tube variability in PCR amplification (Gilliland et al., 1990), it now appears that thermal cycler performance is the key determinant of yield, reliability and reproducibility of PCR amplification (Hoof et al., 1991). Dr. Paul Murphy's experience in this field was invaluable. The results of experiment 1 illustrate that the establishment of a PCR protocol for a specific gene is equally as important as the hardware in producing reliable results. For the protocols used in this chapter, there is a direct relationship between input mRNA and final PCR product, clearly distinguishing 3-fold differences in the starting concentration of bFGF mRNA.

We have applied semi-quantitative RT-PCR analysis to the detection of bFGF and FGF receptor mRNAs in discrete regions of the hypothalamic-pituitary axis in individual rats. In the present study the MBH was the richest source of bFGF mRNA examined, greater than that observed in the cortex and cerebellum whereas the lowest values were localized to the anterior and posterior pituitary. The semi-quantitative estimates of bFGF and FGF receptor mRNA abundance reported here are compatible with recent *in situ* hybridization studies describing the distribution of bFGF and FGF receptor mRNA in various regions of the rat brain (Emoto et al., 1989; Wanaka et al., 1990). The regional expression and

regulation of bFGF in the pituitary appears to be species-specific. Bovine (Baird et al., 1985a) and human (Too et al., 1987) pituitaries are rich sources of bFGF but the level of bFGF in rat pituitary is much lower (Baird et al., 1985b; Baird et al., 1986). Interestingly, as our results confirm, the rat pituitary expresses very low levels of the mRNA for this product. Gonzalez et al. (1992) have suggested that in the rat pituitary stores of bFGF may originate elsewhere, possibly in the hypothalamus.

We employed semi-quantitative RT-PCR analysis to determine changes in the abundance of bFGF and FGF receptor mRNAs in the hypothalamus of female rats in which first ovulation (sexual maturation) has been induced. Two independent methods were used for the induction of precocious first ovulation. First, the effects of daily NMDA injections, which accelerate sexual maturation (MacDonald and Wilkinson, 1990; see Chapter 2) and second, PMSG treatment, a widely used and reliable technique (Wilson et al., 1974; Jacobson et al., 1988).

We observed a significant (50 to 60%) decline in the expression of bFGF mRNA in the MBH of ovulating rats treated with NMDA. A significant reduction was not observed in the MBH of NMDA-treated rats which did not ovulate nor did we see an effect of NMDA in the cerebral cortex of treated rats. In addition, the level of expression of bFGF.R mRNA was not altered by NMDA treatment. These data suggest that the expression of bFGF mRNA is modified coincident with NMDA-induced first ovulation. This is curious as we know from previous work that NMDA activates the hypothalamic-pituitary-ovarian axis even in rats which have not yet reached precocious puberty, as shown by the progressive rise in estrogen-dependent uterine weight (see Chapter 2). Indeed, Table 5 indicates that the uteri of the NMDA (nonVO) rats were stimulated compared to controls. There is some suggestion in Fig. 53A that in this sub-group bFGF mRNA expression is reduced although it does not reach

significance. Based on these experiments, we suspect that bFGF mRNA begins to decline before first ovulation occurs and therefore may be estrogen-dependent. This suggestion is not without precedent as Ma et al. (1994) reported a significant decrease in the expression of EGF receptor on the morning of first proestrus which rebounded at the time of the LH surge (Ma et al., 1994). In future experiments it would be instructuctive to examine the expression of bFGF and its receptor through the pubertal process as per Ma et al. (1994).

In the next step of this project, we used PCR to examine hypothalamic bFGF expression in the PMSG model which is a reliable and nonexcitotoxic method of inducing early puberty. The results were in agreement with what we observed in the NMDA experiment, confirming a significant (40%) decrease in bFGF mRNA on the day of first ovulation in PMSG-treated rats compared to controls. The PCR experiment was repeated a second time using slot-blot hybridization rather than gel electrophoresis with findings of a 50% decrease in bFGF mRNA in the MBH of PMSG-treated rats. The results of these experiments raise the possibility that the change in bFGF expression in the MBH may be the result of an estrogen-dependent process occurring at the time of first ovulation. In order to confirm this suggestion it would be necessary to compare the effect of NMDA and PMSG treatment on the hypothalamic expression of bFGF mRNA in ovariectomized and sham animals receiving oil or estrogen replacement. In addition, if bFGF is one of the components necessary for the final maturation of the reproductive hypothalamus, a reduction in the expression of the bFGF gene may signal the completion of the maturational process.

The immunocytochemistry experiments clearly show the presence of bFGF-li in the MBH, including the ME. These data confirm the results of the RT-PCR determinations. In contrast, however, the bFGF protein levels (bFGF-li) are elevated in the ARCN of NMDA-induced ovulating rats (P29) at a time when

bFGF mRNA levels are reduced. There are at least two possibilities to account for this. First, the half life of bFGF could be significantly longer than that for bFGF mRNA. Second, the induced levels of bFGF may exert a negative feedback on gene expression, such that by P33 bFGF-li is actually lower than control levels. Data from the PMSG experiments are not entirely consistent with this argument since PMSG treatment did not significantly alter bFGF-li in the ARCN of female rats. There was most likely a technical problem with the immunocytochemistry procedure in this experiment. In comparing the control unstimulated animals with the last experiment the cells were very faint and not as distinct as that seen previously. In the densitometric analysis, the threshold was reduced from 200 in the previous experiment to 150 in order to count the cells. It would be beneficial to repeat this experiment and examine bFGF-li at a later time point. Also, this experiment would be more valuable if the expression of bFGF in the MBH and cortex of NMDA- and saline-treated rats was examined over the course of treatment (i.e. at P24, P27, P29 (VO and nonVO), P31 and P33). It would also be useful just to examine the ontogeny of bFGF expression in the MBH and cortex of intact control rats from P2 to P40 and on the day of VO. Others have examined the ontogeny of bFGF expression in whole brain which appears to peak at approximately puberty (Caday et al., 1990; Riva and Mocchetti, 1991; Giordano et al., 1991) but it has not been examined in the hypothalamus.

Two further practical points need to be made which might conceivably alter the interpretation of the data. First, the control gene (actin) used in the PCR experiments may not be truly static such that puberty induction with NMDA or PMSG may differentially alter its expression. Lustig et al. (1992) have reported that the gene cyclophilin is an appropriate standard for developmental studies as the ratio of cyclophilin to poly-dT50 is constant between 1 and 60 days in male and female rats. The use of this gene as an internal standard has also been

validated by Junier et al. (1992) for use in their puberty studies. Therefore, the constitutively expressed cyclophilin gene may be a better internal standard in our studies than actin.

Second, there are also inherent problems with PCR which make it a difficult tool to use for quantification. For instance, minor differences in DNA concentrations in primary samples will be exponentially increased with each cycle. In addition, as the cycle number increases, the production of new DNA product will decline or plateau as the dNTPs are depleted. With single point analysis (i.e. all samples are run for 25 cycles and then analyzed) it is not possible to separate samples in the linear phase from those that have plateaued. One solution to this is to examine the DNA product at multiple points over time (i.e. @ 10, 12, 14, 16, 18, etc... cycles) and plot the relative quantity of DNA against cycle number. Theoretically, it should be possible to extrapolate back to the 0 cycle to measure the initial concentration of cDNA in a sample. Multiple point analysis has been reported to be more reliable than semi-quantitative single point analysis (Chelly et al., 1988) because it reduces the significance of incongruent data and it eliminates data points in the plateau stage of the reaction. RT-PCR with multiple point analysis may be a more accurate tool for measuring cDNA than the technique used in this chapter.

Our understanding of the mechanism by which NMDA induces precocious puberty in female rats is presently incomplete. Injection of NMDA clearly stimulates the secretion of gonadotrophins (MacDonald and Wilkinson, 1992). However we have been unable to reproduce the effect of NMDA on puberty by using an equipotent dose of LHRH (see Chapter 2). This suggests that precocious maturation may be mediated by another hormone, for example prolactin, or by a direct influence of NMDA on the central nervous system (CNS). NMDA is reported to modify synaptic structure *in vivo* (Brooks et al.,

1991) and the growth/differentiation of neurons *in vitro* (McDonald and Johnston, 1990). The NMDA subtype of the glutamate receptor has also been implicated in the regulation of nerve growth factor production *in vitro* (Amano et al., 1992). In contrast, NMDA can act as a neurotoxin at sufficiently high doses, and bFGF is reported to protect against this effect (Freese et al., 1992; Nozaki et al., 1993a,b; Frim et al., 1993).

Altered expression of bFGF in the MBH following the NMDA regimen used to induce early puberty may be part of a neuroprotective process. We know from chapter 3 that there is a rapid and transient increase in immediate early gene expression in the MBH (as well as other CVOs) following NMDA treatment. The likely hypothalamic locus of the NMDA (and PMSG) effects on puberty is supported by our observation that cortical bFGF mRNA is unaffected. Chapter 4 illustrated that for at least 7 days subsequent to glutamate agonist treatment, cells within the ARCN express the inducible heat shock protein, HSP72. If the cells within the ARCN are injured or stressed by treatment with the excitotoxin, as is suggested by HSP72-li in the MBH, bFGF expression may be enhanced in a programmed healing process to prevent neuronal degeneration, promote neurite outgrowth and stimulate the proliferation of glial cells. In fact, bFGF has been shown to have potent neurotrophic effects on immature hypothalamic neurons in culture at concentrations as low as 15 pg/ml (Torres-Aleman et al., 1990). Speculating further, it is plausible that NMDA may increase synaptogenesis and even mitogenesis in the MBH via a growth factor such as bFGF because of its mild excitotoxic properties at this low dose. Although it is not neurotoxic, PMSG is able to stimulate synaptogenesis in the hypothalamus (Matsumoto and Arai, 1977) and we have also shown that PMSG-induced first ovulation can be blocked by the NMDA antagonist MK-801 (MacDonald and Wilkinson, 1990).

The antagonistic properties of bFGF and NMDA in neuronal cytoarchitecture and neurotoxicity have been explored by Mattson's group (Mattson et al., 1989; Mattson and Rychlik, 1990; Mattson et al., 1993). The mechanism by which FGF opposes the neurodegenerative effects of glutamate may be linked to a mechanism involving protein synthesis and the reduction of glutamate-induced release of intracellular calcium (Mattson et al, 1989; Mattson, 1990). Mattson et al. (1993) have recently reported that bFGF applied to cultured hippocampal cells reduces the translation and transcription of a NMDA receptor protein (NMDARP-71) and reduces the vulnerability to NMDA-induced neurotoxicity.

The altered expression of bFGF could also be a physiological rather than an injurious response to NMDA and may not be related to synaptogenesis. There is evidence in the literature which indicates that bFGF may have a neuroendocrine role. bFGF has been shown to increase the release of LHRH from hypothalamus *in vitro* (Ojeda et al., 1990). However, the concentrations required were high (100 ng/ml) and it has been suggested that aFGF rather than bFGF is the effective moiety in this regard (Ojeda et al., 1992). Nevertheless, this relatively high concentration (approx 5×10^{-9} M) might mask a lower active concentration because of rapid degradation and general stickiness of the peptide to the culture apparatus. In the bovine but not the rat pituitary, bFGF release can be modulated by KCl and estrogens (Baird et al., 1985a; 1986). In contrast, Schechter and Weiner (1991) reported that silastic implants of estradiol caused pituitary hyperplasia and a concomitant increase in bFGF-li in gonadotropes. bFGF has been localized to human ovarian follicles (Watson et al., 1992), bovine corpus luteum (CL) (Miyamoto et al., 1992) and rat uteri (Wordinger et al., 1992). bFGF has been implicated in the regulation of folliculogenesis (Roy and Greenwald, 1991) and in the secretory function of the CL (Miyamoto et al., 1992).

In addition to its role in the reproductive axis, bFGF has also been implicated in the synthesis and secretion of somatostatin (Zeytin et al., 1988) and as a permissive factor in the cellular action of growth hormone releasing factor (Zeytin and Rusk, 1988). Interestingly, Sasaki et al. (1991) have reported that injections of FGF into the third ventricle decrease food intake in their male Wistar rats, whereas injection of the antibody to bFGF into the lateral hypothalamus increases food intake.

Despite all of the hypotheses, a key experiment which is required to prove a causative role for bFGF in NMDA-induced precocious puberty would involve blocking the action or production of bFGF in the MBH. This would involve stereotaxic implantation of a cannula and infusion of an antisense oligonucleotide (Wagner, 1994) or a bFGF antibody into the MBH or the third ventricle of juvenile female rats undergoing our protocol of precocious first ovulation.

CHAPTER 6 - SUMMARY AND FUTURE RESEARCH

Mammals are unable to reproduce until an undetermined physiological process in the central nervous system initiates pulsatile secretion of LHRH. The *biological clock* which activates pulsatile gonadotropin secretion at puberty remains elusive. It is hypothesized that first ovulation is due to the removal of an inhibitory system, introduction of an excitatory drive, or a combination of the two processes. Many neurotransmitters, neuromodulators and their various receptors have been implicated in the inhibition and/or stimulation of puberty. The complexity of the pubertal process appears to increase with each new publication. The question of how the brain controls the onset of puberty will be answered by many different laboratories over several years of research. This thesis has focused on the role of the neurotransmitter glutamate, acting through its NMDA receptor, in the onset of puberty.

A simple treatment regime involving four to five daily injections of NMDA (s.c.; 20 mg/kg/day) between P21 and P29 reliably accelerates the onset of sexual maturation in female rats. NMDA treatment rapidly releases LH in a dose-dependent manner but the stimulatory effects of NMDA on puberty do not depend on the immediate release of LH. The effects of NMDA on puberty, however, may be partially due to enhanced secretion of prolactin - a hormone known to cause precocious puberty (Advis and Ojeda, 1978). When NMDA is administered between P21 and P25, a three day delay occurs prior to stimulation of the pituitary-ovarian axis (i.e. estrogen-dependent increase in uterine weight). This evidence indicates that NMDA activates a progressive neuronal process which culminates in early puberty. This also suggests that glutamate and the NMDA receptor are not the sole factors controlling sexual maturation. Other

neurotransmitters, and possibly other glutamate receptors, may also be involved in the process of sexual maturation.

An immunocytochemical technique identifying the protein product of the gene *c-fos* was used to identify the site of action of NMDA and MSG in the CNS. FLI was evident in circumventricular organs (ARCN/ME, SFO, AP, and AMPOA at the OVLT) as well as the PVN and SCN 2 hrs. following EAA injection. FLI is a specific but not a sensitive indicator of neuronal activation. In other words, I am confident that FLI following NMDA represents true neuronal activation but the absence of FLI does not equate with lack of stimulation.

It is known that glutamate and its analogues (NMDA, KA, DA) are capable of inducing neuronal injury or death. and that circumventricular organs are particularly susceptible to damage from glutamate (Olney, 1969). To examine whether NMDA or MSG are damaging cells, I localized the product of a stress protein (HSP72) following treatment with EAA. In juvenile animals treated with NMDA, HSP72-li was found in CVOs only and staining was most prominent in the ARCN/ME and AP. In contrast, HSP72 expression was not evident in neonatal animals (P2 to P10) injected with NMDA or MSG. It is possible that the genetic mechanisms transcribing and translating the stress protein are not present until a later stage of development. Alternatively, there may be sufficient damage to these young cells to arrest transcription following the rapid expression of *c-fos*..

NMDA may lead to precocious puberty by removing inhibitory cells or by stimulating an increase in excitation through a reactive growth process as a result of injury. If the cells within the ARCN are injured or stressed by treatment with the excitotoxin, as is suggested by HSP72-li in the MBH, the expression of the growth factor, bFGF, may be enhanced in a programmed healing process to prevent neuronal degeneration, promote neurite outgrowth and stimulate the

proliferation of glial cells. Our data suggests that the expression of bFGF changes with the induction (NMDA or PMSG) of puberty. There is reason to suspect that bFGF, in addition to TGFα and IGF-1 (Ojeda et al., 1992) might serve as a necessary neurotrophic or physiological factor in the control of the onset of puberty. It will be possible, using RT-PCR or *in situ* hybridization to quantify mRNA levels of bFGF and other members of the FGF growth factor family in discrete brain nuclei at various maturational stages to confirm this hypothesis.

There are several further lines of evidence that would be worthwhile pursuing to determine the underlying mechanism of NMDA-induced puberty. Are there are other indicators of NMDA-stimulated synaptogenesis? I have done some preliminary investigations on the possible role of other growth factors (i.e. NGF and TGFα), synapse-specific proteins (i.e. synaptophysin) and neuronal architecture proteins (i.e. microtubule associated proteins, MAPs) in the onset of puberty. Figure 59 illustrates the immunocytochemical localization of several possible markers of altered neural connections. NGF receptor-immunoreactive fibers emerging from tanycytes lining the third ventricle are shown in Fig. 59A (see arrows). Ma et al. (1994) have also demonstrated EGF.R immunoreactive fibers in tanycyte processes of the rat. There are numerous cell bodies (arrowheads), neuronal processes (arrows) but not nuclei (see cells next to stars) labeled for NGF.R in the preoptic area and other areas of the hypothalamus (Fig. 59B, E and F). Fine fibres displaying TGFα-li are also observed surrounding the third ventricle (see arrows; Fig. 59C). A high magnification photomicrograph of synaptophysin-li in the ARCN (Fig. 59G) shows small black dots, possibly synapses, surrounding a clear unlabeled cell body. MAP2 is a dendrite specific protein and, as shown in Fig. 59D and H, is widely distributed in the brain. The juvenile form, MAP2c, can be identified by subtracting the stained areas in D from those in H. Therefore, a significant amount of staining in the median

eminence and in the fibers emanating from the third ventricle are MAP2c-positive.

Matsumoto and Arai (1977) laboriously counted synapses in the ARCN of their rats at puberty and showed a direct correlation between synaptic number and reproductive competence. It would be exciting and challenging to find a structural marker, perhaps synaptophysin or MAP2c, which has a specific configuration at puberty. Interestingly, Crandall et al. (1989) have already reported a distinct pattern of MAP2-li in the ventromedial hypothalamic nucleus which disappears in the third postnatal week. In terms of our puberty model, several reports have implicated glutamate and the NMDA receptor in the control of MAP expression (Bigot and Hunt, 1990, 1991; Bigot et al., 1991; Halpain and Greengard, 1990). Therefore, the neuronal "appearance" of puberty may be readily attainable.

Figure 59. - Immunocytochemical localization of various growth factors and cytoskeletal proteins in the CNS of juvenile female rats.

Photomicrographs of various growth factors, synapse-associated proteins and microtubule associated proteins in the hypothalamus and preoptic area of juvenile female rats. They represent possible markers of the structural changes occurring in the hypothalamus at puberty. A,B,E,F: NGF receptor in ARCN/ME, diagonal band of Broca, lateral hypothalamus, vertical band of Broca, respectively; C: TGFα in ARCN/ME; G: synaptophysin in ARCN; D: MAP2 in ARCN/ME; H: MAP2c in ARCN/ME Scale bar = 100 μm.
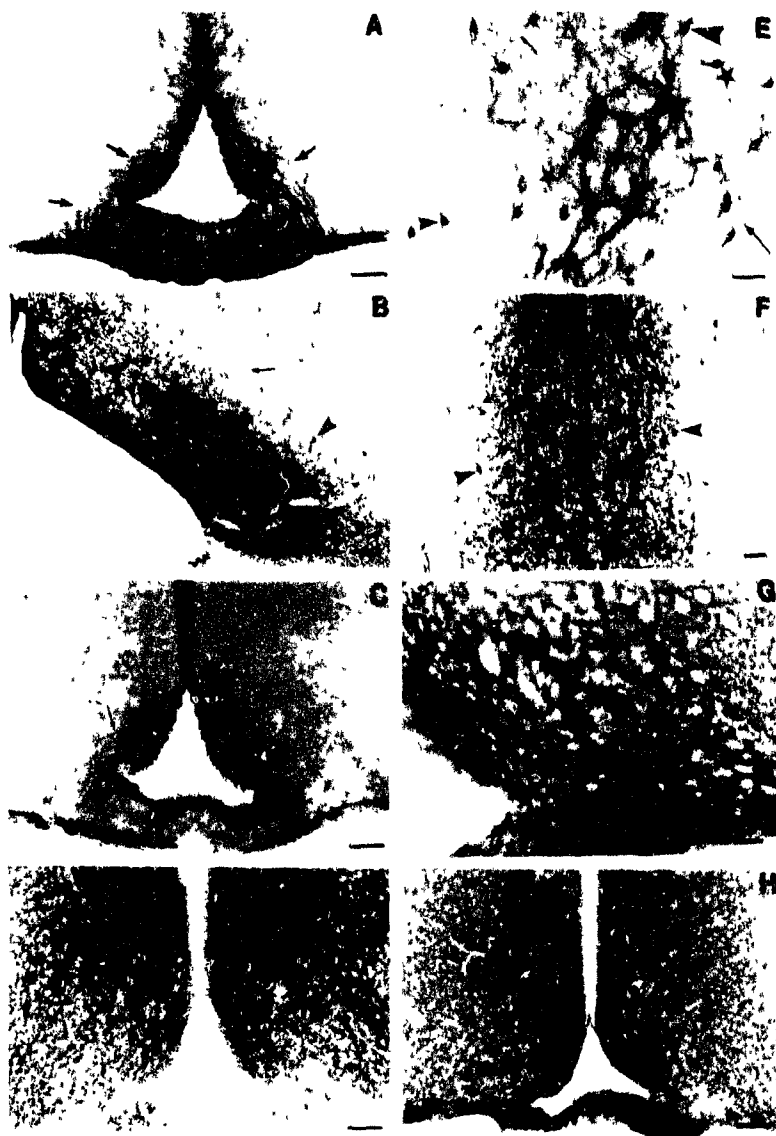
Fig. 59

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