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Sexually dimorphic effects of NMDA receptor antagonism on brain–pituitary–gonad axis development in the platyfish

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Abstract

The *N*-methyl-D-aspartate glutamate receptor (NMDAR) is found in hypothalamic nuclei involved in the regulation of reproduction in several species of mammals and fishes. NMDAR is believed to affect reproductive development and function by regulating gonadotropin releasing hormone (GnRH)-producing cells. These pathways are likely to be sexually dimorphic, as are several other neurotransmitter systems involved in reproductive function. In this report, male and female platyfish received intraperitoneal injections of 0, 5, 10, 20, 40 or 60 μg/g body wt. of the non-competitive NMDAR antagonist MK-801. Injections began at 6 weeks of age and continued thrice weekly until control animals reached puberty, as evidenced by anal fin maturation. The percent of pubescent animals was significantly affected by sex and treatment, with fewer MK-801-injected females in puberty than control females at each dose ($P < 0.001$), and fewer pubescent females than males at 10, 20 and 40 μg/g ($P < 0.05$). There were no MK-801-related effects in males. Histological analyses revealed typical immature gonads and pituitary glands in treated females, and typical mature morphology in control females and all males. Immunocytochemical distribution of the R1 subunit of the NMDAR within the brain-pituitary-gonad (BPG) axis was limited to GnRH-containing brain cells in all animals; however, NMDAR1 distribution was in an immature pattern in treated females and a mature pattern in all others. Neural concentrations of GnRH were unaffected by MK-801 treatment in both sexes. These data suggest that in the platyfish, NMDAR influence on reproductive development is sexually dimorphic and occurs at, or above, the level of GnRH-containing cells of the BPG axis.

Keywords: Glutamate; Fishes; Puberty; Reproduction; Gonadotropin releasing hormone; MK-801; NMDA; Sexual dimorphism; Immunocytochemistry

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1. Introduction

Glutamate is known to contribute to the neuroendocrine regulation of reproduction in mammals, in part through hypothalamic *N*-methyl-D-aspartate receptors (NMDAR) (Brann and Mahesh, 1997, for review). Presumably, NMDAR influence the pulsatile release of gonadotropin-releasing hormone (GnRH) required for the initiation of puberty and the maintenance of reproductive capability (ibid.). NMDAR have been localized in GnRH-containing nuclei in non-mammalian vertebrates, including several species of fishes (Monaghan and Maler, 1991; Flett et al., 1994; Flynn et al., 1997; Bottai et al., 1997), suggesting NMDAR influences on reproduction in these species as well.

The platyfish model (*Xiphophorus maculatus*) is particularly well-suited for studying age- and gender-related events in the reproductive system. Platyfish have a sexually dimorphic anal fin which becomes more conspicuous at puberty, when an androgen-dependent metamorphosis in the male produces an intromittent organ for copulation, the gonopodium (Schreibman and Kallman, 1978). The development of the gonopodium takes place in a well-defined, step-wise manner which correlates with neuroendocrine and reproductive system development and therefore allows identification of animals by sex and developmental stage. In females, there is no change in the anal fin, although development of the neuroendocrine and reproductive systems occurs with similar timing. As in mammals, maturation in platyfish depends on stepwise developmental events in GnRH centers in the brain and gonadotropin (GtH)-producing cells in the pituitary gland, culminating in an adult gonad capable of producing functional gametes (Schreibman et al., 1991 for review).

Our laboratory has shown a sequential appearance of salmon (s), mammalian (m), chicken II (c), and lamprey I (l) forms of the GnRH decapeptide in three neuroendocrine brain nuclei and then in the pituitary gland of the platyfish (Magliulo-Cepriano et al., 1994). These nuclei are the bilateral nucleus olfactoretinalis (NOR) located at the base of the telencephalon, the nucleus preopticus periventricularis (NPP), and the nucleus lateralis tuberis (NLT). The sequential appearance of GnRH in these neuroendocrine nuclei correlates with stage of development and not with chronological age (Halpern-Sebold et al., 1986), supporting the idea that GnRH initiates developmental changes in the platyfish reproductive system. The GnRH system, however, is believed to be mature before the onset of puberty; in monkeys and platyfish GnRH-containing neurons have been identified in immature animals (Halpern-Sebold and Schreibman, 1983; Goldsmith and Song, 1987). It is likely, therefore, that altered neurotransmitter input to GnRH-containing cells is ultimately responsible for the increase in pulsatile GnRH that results in adult reproductive function.

Sexual dimorphisms in neurotransmitter function have been well-established in several species and several systems, including GnRH immunoreactivity in the musk shrew brain (Rissman and Li, 1998), NMDAR modulation of dopamine concentrations in rat brain (Wagner et al., 1993), and developmental changes in somatostatin distribution in the platyfish brain (Margolis-Nunno et al., 1987). While there are suggestions of gender-related differences in NMDAR influence on the BPG axis, no single study has systematically examined NMDAR-related effects on reproductive development in males and females. In the current study, the effects of long term NMDAR antagonism on maturation of the BPG axis in male and female platyfish are reported.

2. Methods

2.1. Animals

Male and female platyfish were derived from genetically defined stocks (JP 163) which originated at the Genetics Laboratory of the Osborn Laboratories of Marine Sciences at the New York Aquarium for Wildlife Conservation. These stocks have been maintained in our laboratory since the 1970s and many have been inbred for more than 80 generations by brother and sister matings. For this study, all animals were kept at a ratio of 1 fish/gallon of aged tap water, in aquaria containing plants, gravel and snails, at 27°C. They received 16 h of artificial light per day (7.00–23.00 h) and were fed a beef liver–cereal paste or live brine shrimp nauplii, supplemented by dried flake food three times per day. Animals were an early maturing (EM) genotype that begin puberty at approximately 8 weeks of age and are completely mature at 3–4 months (Kallman et al., 1973).

A total of 92 platyfish from four broods born on 6/6/96 ($n = 24$; 10 males and 14 females), 7/12/96 ($n = 23$; 9 males and 14 females), 7/21/96 ($n = 14$; 7 males and 7 females), and 4/10/97 ($n = 31$; 20 males and 11 females) were separated by sex and into control and experimental groups at 4 weeks of age. Treatment began at 6 weeks of age and continued until the first control male was sexually mature, which varied from 14 to 21 weeks of age, and was judged by complete metamorphosis of the gonopodium (see Section 1). All animals were anesthetized 3 mornings/week between 10.00 and 12.00 h by immersion in a 0.04% solution of tricane methane sulfonate (MS-222; Sigma Chemical Co., St. Louis, MO, USA). Intraperitoneal (i.p.) injections of 5 μl of MK-801 (dizocilpine maleate [10,11-dihydro-5-methyl-5-hydrogen maleate]; Research Biochemicals International, Natick, MA, USA) in saline, or physiological saline solution alone, followed. MK-801 concentrations were maintained at 5, 10, 20, 40 or 60 μg/g by calculating mean body weight for each group once each week.

All animals were sacrificed by decapitation between 2 and 3 h after the final injection, and staged at autopsy for gross gonadal development and anal fin metamorphosis (see Section 1). Immature animals were defined as those with small, transparent, and undifferentiated gonads and no metamorphosis of the anal fin in males. Mature animals were defined as those with large and fully mature gonads: female ovaries contained yolky oocytes and males had large, translucent, sperm-containing testes and a fully differentiated anal fin (i.e. stage 6 gonopodium).

The number of mature and immature animals at each sex and each MK-801 dose were compared with two-way analysis of variance (ANOVA). Body weights from the first and eighth week of injections were used to calculate percent increase in weight for the control group and each MK-801-treatment group, and these data were also analyzed with two-way ANOVA. If differences were significant at $P < 0.05$, Student–Newman–Keuls post-hoc analyses were applied with $P < 0.05$ again required for significance.

2.2. Histology and immunocytochemistry

Heads and gonads from at least two animals in each group were post fixed in Bouin solution under vacuum immediately after sacrifice, decalcified (S/P Decalcifying Solution; Baxter, McGaw Park, IL, USA), dehydrated in a graded ethanol and butanol (Zirkle) series, and embedded in Polyfin (Triangle Biomedical Supplies, Durham, NC, USA). Five micrometer, serial, sagittal sections were mounted on gelatin-coated slides.

Every other section of gonad, pituitary, and NOR was stained with Masson's trichrome procedure for cytological evaluation; the remaining sections were used for immunocytochemical (ICC) analysis with the avidin–biotin method (Vectastain-Elite; Vector Laboratories, Burlingame, CA, USA) as modified for our material (Margolis-Kazan et al., 1981). Sites of antigen localization were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO, USA) in Tris buffer with 0.0125% H_2O_2 at pH 7.4. Antisera to a synthetic peptide corresponding to the C-terminus of the R1 subunit of the NMDA receptor (anti-NMDAR1; Chemicon, Temecula, CA, USA) was used at a 1:200 dilution following a serial dilution study. As characterized by the manufacturer, anti-NMDAR1 recognizes the four most common splice variants of the R1 subunit and does not cross react with any other glutamate receptor subunits.

Control procedures included the replacement of the primary antibody with normal rabbit serum, the elimination of one of the sequential steps in the ICC procedure, and the pre-absorption of anti-NMDA R1 with a synthetic fragment of the receptor subunit (provided by Dr R. Wenthold, National Institutes of Health). All control procedures resulted in the elimination of the immunoreaction.

2.3. Radioimmunoassay

Brains of 3–4 males and females injected with 60 μg/g MK-801, and brains of 3–4 control males and females were pooled and frozen on dry ice immediately after sacrifice and stored for not more than 12 months at −80°C. Tissues were suspended in ice cold 2 M acetic acid, homogenized at one half maximal setting for 15 s using a Polytron PT-45 Homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA), centrifuged at

10 000 × *g* for 45 min at 4°C, and the supernatant was lyophilized overnight (SpeedVac Concentrator SVC 100H; Savant Instruments Inc., Farmingdale, NY, USA). The remaining pellet was reconstituted in water and centrifuged at 14 000 × *g* for 30 min at 4°C; the supernatant was then stored at −80°C for no more than 6 months. Samples were rehydrated in duplicate in phosphate buffered saline (pH 7.0), incubated at room temperature for 30 min with a 1:40 000 dilution of anti-mammalian GnRH (mGnRH) (Peninsula Laboratories, Belmont, CA, USA), then incubated at 4°C overnight with a 1:10 000 counts/min solution of iodinated [^{125}I] mGnRH as previously documented (Sower et al., 1993). Binding was terminated by the addition of dextran-coated charcoal (Sigma Chemical Co., St. Louis, MO, USA), samples were centrifuged at 4000 × *g* for 15 min at 4°C, and sample radioactivity was determined with the LKB Wallac 1282 Compugamma Gamma Counter (LKB Nuclear Inc., Gaithersburg, MD, USA). All samples were counted twice.

The amounts of GnRH in pg/mg brain tissue were calculated from readings in counts/min from one sample of pooled tissue for control males, one for control females, one for MK-801-treated males, and one for treated females. GnRH concentrations were extrapolated from a standard curve counted with the samples. Data were compared using two-way ANOVA with $P < 0.05$ required for significance.

3. Results

3.1. Puberty

There was a significant interaction between treatment and sex on the percent of animals reaching puberty ($F_{1,91} = 8.931$, $P < 0.001$) (Fig. 1). Post-hoc analyses showed differences between females in each of the 5 MK-801-treated groups and control females ($P < 0.001$), and between males and females in the control, 10, 20 and 40 μg/g groups ($P < 0.05$). There were no significant differences between any male treatment group and control males.

3.2. Gonad and pituitary gland histology

Ovarian tissues from control females were typical of mature ovaries, with many large, round oocytes filled with yolk and smaller follicular cells surrounding the mature ova. MK-801-treated females had typical immature ovaries, with few developing oocytes containing small amounts of deposited yolk, and many follicular cells surrounding abundant immature ova. Testicular tissue from control and MK-801-treated males possessed abundant seminiferous tubules containing mature sperm as well as spermatozoa at all developmental stages (data not shown.)

Mid-sagittal pituitary sections from control females contained large regions of gonadotropic

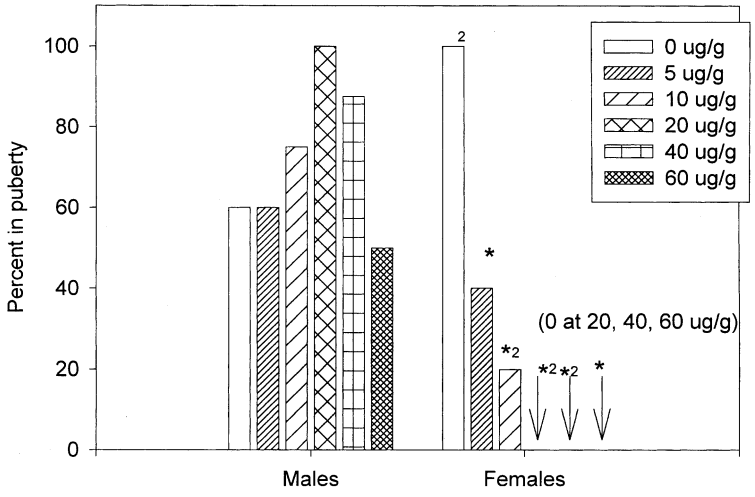

Fig. 1. Sexually dimorphic effect of MK-801 on percent of animals in puberty. Percent of male and female platyfish in puberty (as described in Section 2) after 8–15 weeks of thrice weekly i.p. injections of saline or MK-801. * = significantly different from same sex control group, $P < 0.001$. 2 = significantly different from same treatment males, $P < 0.05$.

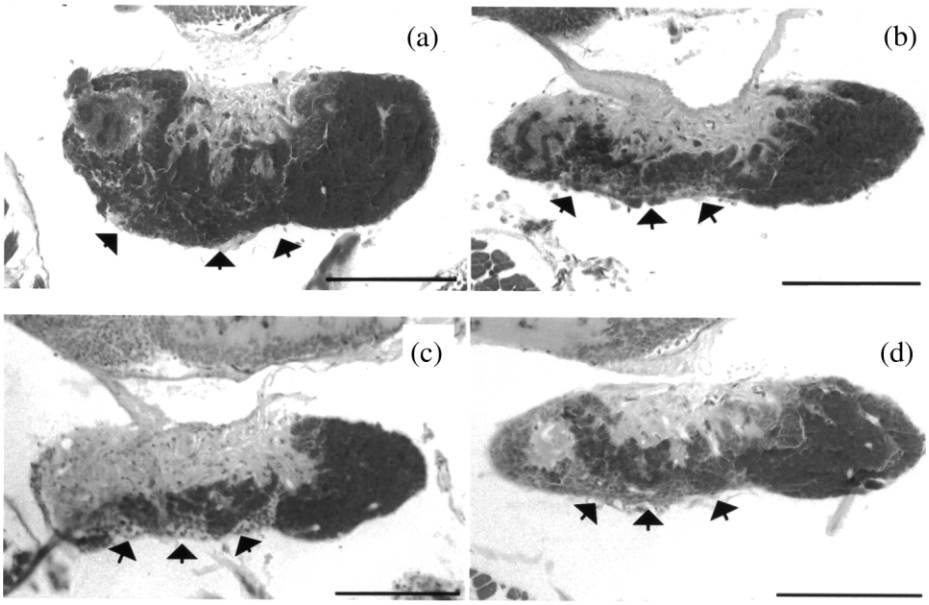

Fig. 2. Effects of 60 μg/g MK-801 on pituitary histology. Mid-sagittal sections stained with Masson's trichrome. (a) A control female. The ventral gonadotropic zone is large with a layer of small, darkly stained, ovoid cells (arrowheads). (b) An MK-801-treated female. The ventral gonadotropic zone is undeveloped (arrowheads). (c) A control and (d) an MK-801-treated male. The ventral gonadotropic zones are of intermediate thickness with some darkly stained, ovoid cells (arrowheads) in both sections. Bars = 100 μm.

cells, with many layers of small darkly stained ovoid cells visible on the ventral surface of the gland (Fig. 2a). In treated females there was little proliferation of the gonadotropic zone; a thin layer of cells was present on the ventral surface of the pituitary (Fig. 2b). Pituitary tissue from control and treated males contained developing gonadotropic zones with evidence of cell proliferation characteristic of a mature gland (Fig. 2c,d).

3.3. NMDAR1 distribution

Immunocytochemistry (ICC) of the gonad revealed the lack of immunoreactive (ir-) NMDAR1 in males or females of any group (Table 1). ICC of the pituitary (mid-sagittal sections) showed ir-NMDAR1 localization in cell bodies of the rostral pars distalis (composed essentially of prolactin cells) in all animals; there was also non-specific staining in the caudal pars distalis, but not in the GtH-producing cells of the gonadotropic zone. Sections of the NOR from all animals revealed ir-NMDAR1 in the cytoplasm of ovoid cells with large, round unstained nuclei (Fig. 3a–d). Intensity of staining was equal in control males and females, slightly attenuated in MK-801-treated males, and greatly attenuated in treated females (Table 1).

3.4. GnRH concentration

There was no treatment by sex interaction and

Table 1

	Control male	Control female	Treated male (60 μg/g MK-801)	Treated female (60 μg/g MK-801)
NMDAR1 localization				
Gonads	–	–	–	–
Pituitary (gonadotropic zone)	–	–	–	–
Nucleus olfactoretinalis	+ + +	+ + +	+ +	+
Neural concentration of GnRH	0.68 ± 0.12	0.165 ± 0.015	0.63 ± 0.25	0.27 ± 0.04

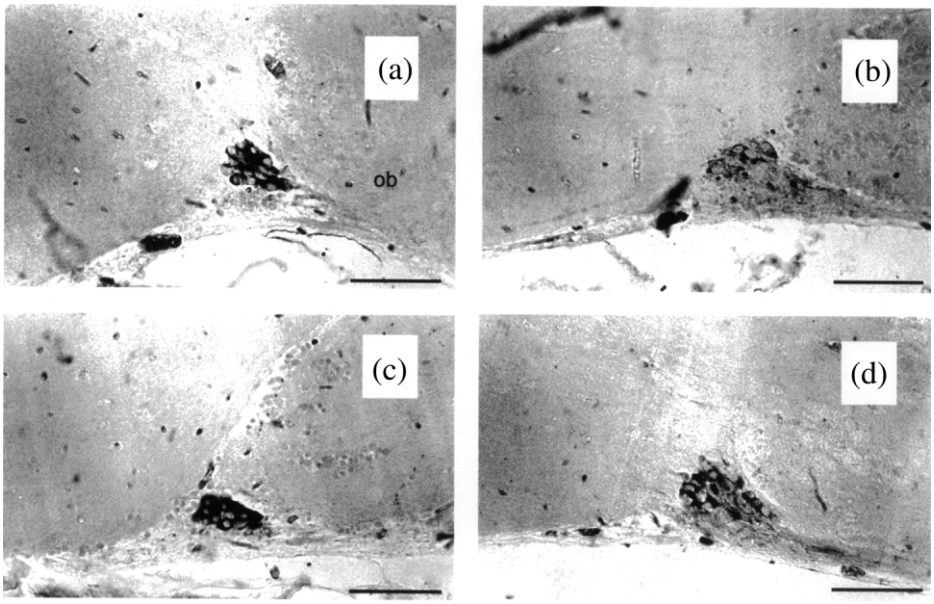

Fig. 3. Sexually dimorphic effect of 60 μg/g MK-801 on NMDAR1 expression in the NOR. Sagittal NOR-containing sections stained with anti-NMDAR1. (a) A control female. Large cells with darkly stained cytoplasm and clear nuclei just dorsal to the olfactory bulb (ob) define the NOR. (b) An MK-801-treated female. Scattered staining in cell bodies of the NOR. (c) A control and (d) an MK-801-treated male. Cytoplasm of NOR cells are darkly stained in both samples. Bars = 25 μm.

no overall effect of treatment on the concentration of GnRH in neural tissue as determined by radioimmunoassay (RIA) (Table 1). There was a significant effect of sex, with a higher concentration of GnRH in males compared to females ($F_{1,7} = 9.725$, $P < 0.04$).

3.5. Weight gain

There was a significant overall effect of treatment on the percent increase in weight during the first 8 weeks of MK-801 treatment ($F_{5,11} = 5.144$, $P < 0.05$), but post-hoc analyses indicated that no treatment group differed from the control group (Fig. 4a). There was no significant treatment by sex interactions, and no overall effect of sex (Fig. 4b).

4. Discussion

These data show that chronic NMDAR antagonism with MK-801 inhibited the initiation of puberty in female platyfish. This effect was dose-dependent; the percent of pubescent females decreased with increasing concentrations of MK-801, and at the highest doses, no treated females showed any signs of sexual maturity. By comparison, the same NMDAR antagonism had no effect on the initiation of sexual maturity in males. Histological and ICC analyses showed an inhibition of maturation in the gonad, the pituitary gland, and the NOR of MK-801-treated females, and no inhibition in the BPG axes of males or saline-treated females; RIA analyses showed no MK-801 effect on neural concentration of GnRH in either sex, and analysis of weight gain showed a similar decrease in both sexes. Together, these data suggest an NMDAR influence on the timing of puberty in female platyfish.

Our data reveal some inhibition of weight gain by MK-801 treatment. Since a minimum body weight is required for puberty initiation (reviewed in Frisch, 1990), this is a possible mechanism of the MK-801 effect on puberty that we report. However, despite an overall effect of MK-801 treatment, our analyses showed no differences between any treated groups and controls, raising the possibility that this was not a true effect. A recent report that MK-801 treatment resulted in *increased* weight gain in rats (Jahng and Houpt, 2001) also makes it unlikely that MK-801 would decrease weight gain here. Finally, there is the discrepancy between the similarly decreased

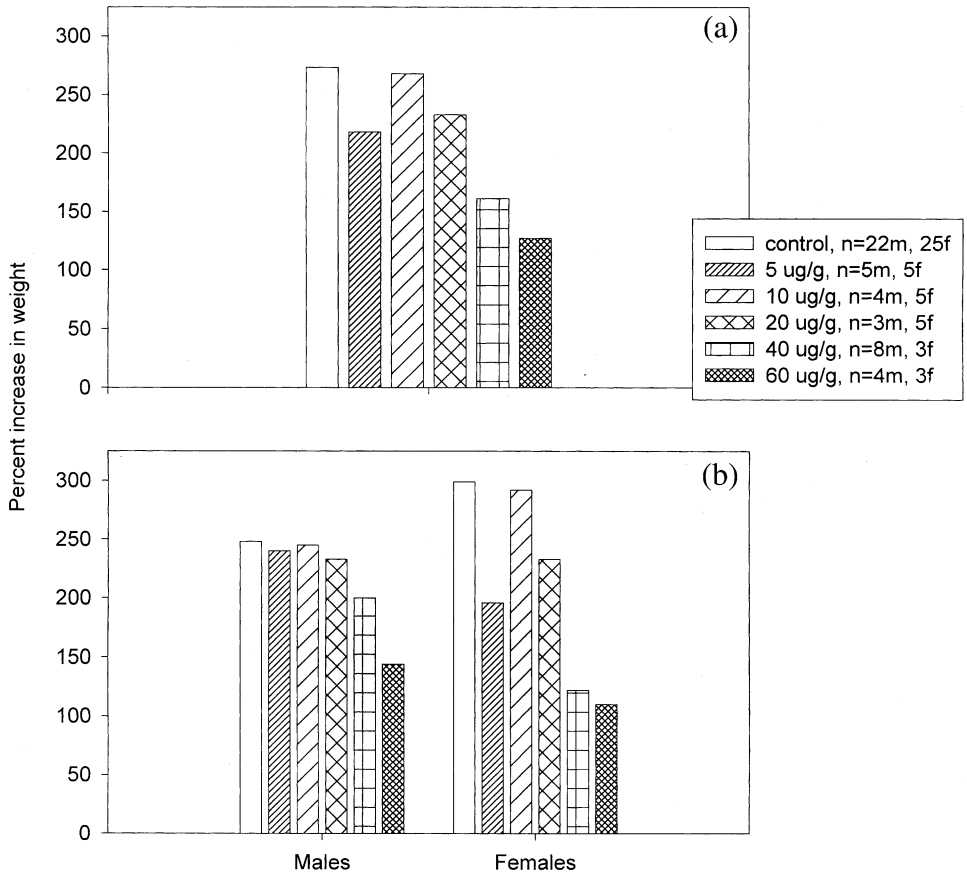

Fig. 4. Effect of MK-801 on weight gain. Mean percent increase in weight after 8 weeks of thrice weekly i.p. injections of saline or MK-801. (a) There is a significant overall effect of treatment ($P < 0.05$), but no treated group differs from the control group. (b) There is no significant sex by treatment interaction, and no overall effect of sex.

weight gain in males and females and the sexually dimorphic decrease in puberty attainment. While this might suggest a sexually dimorphic sensitivity of the BPG axis to body weight, it is also suggestive of a sexually dimorphic NMDAR influence on the platyfish BPG axis.

Puberty inhibition in female platyfish by NMDAR antagonism is consistent with observations in other species. In female rats, NMDAR antagonism inhibited puberty and NMDAR stimulation induced puberty (Urbanski and Ojeda, 1990; Gore et al., 1996). In female goldfish and rainbow trout, treatment with an NMDA agonist increased serum concentrations of GtH-II (Trudeau et al., 1993; Flett et al., 1994). There are no reports of NMDAR effects on puberty in male fishes, but NMDAR stimulation elicited GtH and GnRH release in rats and monkeys, and inhibited the release of these hormones in guinea pigs and sheep, suggesting a gender- and species-specific NMDAR influence on puberty (Gay and Plant, 1987; Estienne et al., 1989; Farah et al., 1991; Giri and Kaufman, 1995).

NMDAR distribution in fishes has been reported in GnRH-containing areas, such as the NOR in the platyfish, and the diencephalon in the electric fish *A. leptorhynchus*, (Bottai et al., 1997; Flynn et al., 1997, 1999). The identification of a C2′ receptor sequence in Xenopus (Soloviev et al., 1996), when a C2 mammalian sequence was used to prepare our antibody, raises the possibility that we did not see a specific response to the platyfish antigen. While only a Western blot analysis on proteins from platyfish brain tissue would be conclusive, the statement by Soloviev et al. that the Xenopus R1 subunit, '...shares high sequence similarity with mammalian NR1 subunits' is strong support for a specific reaction. Our control procedures, including pre-absorption of the antibody with a synthetic sequence, further

suggest specificity. The sum of the evidence, therefore, suggests that NMDA receptors are present in GnRH-containing systems in fishes, as they are in mammals [reviewed in (Brann and Mahesh, 1997)].

The typically immature pattern of NMDAR1 distribution that we report in the NOR of treated females suggests that MK-801 inhibition occurs above the level of these GnRH-containing cells. While only a crude estimate of quantity is possible with ICC, the representative sections in Fig. 3 clearly show attenuated NMDAR1 staining in the GnRH-containing nucleus olfactoretinalis of MK-801-treated females. Taken together with our previous reports of sexually dimorphic NMDAR distribution and characterization in platyfish (Flynn et al., 1997, 1999), and with the clear sexual dimorphism in puberty onset following NMDAR antagonism that we report here, these ICC data offer further evidence of NMDAR influence on GnRH cells. In addition, the regulation of puberty by way of regulating input to the GnRH system is believed to occur in mammals where GnRH neurosecretory cells are mature before puberty onset (Goldsmith and Song, 1987). The limited NMDAR1 distribution in the pituitary gland of the platyfish, to cells of the rostral pars distalis, an area that does not contain GtH (Margolis-Kazan et al., 1981), is similar to the limited distribution in mammals, and suggests that a direct NMDA effect on GtH is unlikely (Petralia et al., 1994). There are no reports of NMDAR localization in gonadal tissue in mammals nor fishes, and in this study we found no evidence of the NMDAR1 protein in ovarian or testicular tissue of any animals, making a direct gonadal NMDA effect also unlikely.

The absence of an MK-801 effect on neural GnRH concentration that we report must be looked at cautiously as the number of animals assessed was extremely low due to technical problems that resulted in lost samples. Our data, however, were statistically sound, and the lower GnRH that we report in females compared to males agrees with reports in other fish species, such as the ballan wrasse (*Labrus berggylta*), where males had more GnRH-positive cells than females, and the spotted ratfish (*Hydorlagus colliei*), where serum GnRH levels varied in females to lows of less than 60% of male values (Lovejoy et al., 1993; Elofsson et al., 1999). Since we do not see increased neural GnRH in immature (MK-801-treated female) animals, this suggests either a release of GnRH from the hypothalamus to the pituitary gland with attainment of puberty blocked further 'downstream,' or no synthesis of the higher amounts of GnRH that are associated with puberty.

Given the typically immature tissue in the gonad, pituitary gland, and NOR, female platyfish treated with MK-801 appear to have delayed puberty because NMDA receptors located *above* the level of the NOR were inhibited. This interpretation is in agreement with reports in other animals which show that NMDAR in the brain modulates GnRH cells in the hypothalamus to influence adult reproductive system function as well as the developmental changes in the BPG axis that culminate in puberty. We now extend prior reports by systematically comparing males and females to confirm our earlier report of sexual dimorphism in NMDAR regulation of platyfish reproductive development.

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