Prevention of Latently Expressed CYP2C11, CYP3A2, and Growth Hormone Defects in Neonatally Monosodium Glutamate-Treated Male Rats by the N-Methyl-d-Aspartate Receptor Antagonist Dizocilpine Maleate

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ABSTRACT
Neonatal administration of monosodium glutamate (MSG) can produce latently expressed defects in drug metabolism and growth hormone secretion as well as stunted growth and obesity. Instead of secreting growth hormone in the masculine episodic profile, plasma hormone levels are generally undetectable in affected adult male rats. Moreover, male-specific isoforms of cytochrome P450 (P450; e.g., CYP2C11 and CYP3A2), whose combined levels comprise the bulk of the total hepatic P450 in adult male rats, are similarly undetectable in these animals. Since “signalizing elements” in the masculine episodic growth hormone profile are solely responsible for the elevated characteristic male-like expression levels of CYP2C11 and CYP3A2, suppression of the isoforms in the MSG-treated rats appeared to be caused by the simple absence of the hormone from the circulation. However, the reported failures of restored physiologic masculine growth hormone profiles to correct the P450 defects suggested the occurrence of direct MSG-induced liver damage independent of the well known hypothalamic lesions produced by the amino acid. Concurrent administration of dizocilpine maleate (MK-801), a selective and highly potent noncompetitive N-methyl-D-aspartate receptor antagonist of glutamate, completely prevented the adverse effects of neonatal MSG treatment on P450 expression, growth hormone secretion, and growth parameters, indicating that the amino acid-induced defects are solely a result of neuronal (i.e., hypothalamic) damage produced at the time of MSG exposure. The irreversibility of the P450 damage is described as resulting from secondary defects initially induced by the neuronal lesions.

Neonatal exposure to the N-methyl-d-aspartate (NMDA) receptor agonists glutamate and aspartate, excitatory amino acid neurotransmitters, can induce a syndrome characterized by stunted growth and obesity. As adults, affected animals exhibit delayed abnormalities in both growth hormone secretion and cytochrome P450 (P450) expression (Shapiro, 1992; Agrawal and Shapiro, 1997). Circulating growth hormone profiles in rats as well as other species have been shown to be sexually dimorphic (Shapiro et al., 1995). Male rats secrete growth hormone in episodic bursts (~200 to 300 ng/ml of plasma) every 3.5 to 4 h. Between the peaks, growth hormone levels are undetectable. In females, the hormone pulses are more frequent and irregular and are of lower magnitude than those in males, whereas the interpulse concentrations of growth hormone are always measurable (Shapiro et al., 1989; Pampori et al., 1991a). These gender differences in the circulating growth hormone profiles are responsible for the dimorphisms in at least a dozen sex-dependent isoforms of P450 observed in rats and mice (Legraverend et al., 1992; Shapiro et al., 1995).

In the case of CYP2C11, the dominant male-specific isoform comprising ~50% of the total hepatic P450 in the male rat (Morgan et al., 1985), a female-like pattern of continuous growth hormone secretion completely blocks expression of the isoform, whereas total growth hormone depletion from the circulation allows CYP2C11 expression at 20 to 30% of intact male levels (Legraverend et al., 1992; Pampori and Shapiro, 1996). Whereas normal expression of CYP2C11 requires exposure to the episodic “on/off” masculine growth hormone profile, the profile need only be minimal. That is, in contrast to the physiologic masculine hormone profile, full expression of CYP2C11 requires exposure to plasma pulses of only 25 ng/ml [or much less (Agrawal and Shapiro, 2000)], at least once, or perhaps less than once every 12 h, representing <2 to 3% of the masculine physiologic growth hormone secretory output. Irrespective of the pulse amplitudes, the inductive “signal” in the masculine profile is a minimum growth hormone-devoid interpulse of between 100 and 140 ng/ml.
min for full CYP2C11 expression (Waxman et al., 1991; Agrawal and Shapiro, 2001). Another male-specific isoform, CYP3A2 (as well as CYP2A2), is maximally expressed in the growth hormone-depleted, hypophysectomized rat, is somewhat suppressed by physiologic pulse amplitudes in the masculine episodic hormone profile, but completely disappears when exposed to a continuous pattern of growth hormone secretion representing <3% of the normal feminine profile (Pampori and Shapiro, 1996).

As adults, male rats neonatally treated with MSG (4 mg/g of body weight) have neither detectable levels of circulating growth hormone nor measurable concentrations of hepatic CYP2C11 and CYP3A2 (Shapiro et al., 1989; Pampori et al., 1991b). Since MSG destroys differentiating hypothalamic nuclei destined to regulate growth hormone secretion (Millard et al., 1982; Bloch et al., 1984), it has seemed reasonable to conclude that the delayed suppression of CYP2C11 and CYP3A2 is due solely to the permanent absence of growth hormone from the circulation (Pampori et al., 1991b). However, restoration of physiologic masculine plasma growth hormone profiles to neonatally MSG-treated, growth hormone-depleted adult male rats could not restore normal expression levels of CYP2C11 and CYP3A2; this is in contrast to the complete effectiveness of the growth hormone treatment when given to hypophysectomized rats (Shapiro et al., 1993; Waxman et al., 1995). The abnormal responsiveness of P450 isoforms to growth hormone regulation may have resulted from MSG-induced defects in hepatic development independent of the hypothalamic damage produced by the amino acid. To test this hypothesis, we selectively blocked the neuronal effects of MSG by simultaneously administering the noncompetitive NMDA receptor blocker dizocilpine maleate [MK-801 (Wong et al., 1988)] and determined subsequent CYP2C11 and CYP3A2 expression levels and circulating growth hormone profiles in adulthood.

**Materials and Methods**

**Animals.** Animals were housed in the University of Pennsylvania Laboratory Animal Resources facility under the supervision of certified laboratory animal medicine veterinarians and were treated according to a research protocol approved by the University’s Institutional Animal Care and Use Committee. At all times, animals were housed on hardwood bedding in plastic cages, with water and commercial rat diet supplied ad libitum. The animal quarters were air conditioned (20–23°C) and had a photoperiod of 12 h of light, 12 h of darkness (lights on at 8:00 AM). After a 2- to 3-week acclimation period in our facility, the animals were bred by randomly housing two adult female Sprague-Dawley rats [Crl:CD(SD)BR] with an individual adult male of the same strain. On the day of parturition, all the pups in a litter were injected s.c. with either 2 or 4 mg/g of body weight). Recovery from unconsciousness was indicated by the full restoration of the righting response, defined as the ability of the animal when placed on its back on a flat surface to snap over its paws three times within 15 s (Shapiro et al., 1989).

**Sleeping Times.** At 5 to 6 months of age, barbiturate-induced sleeping times were measured in some of the male rats, representing all treatment groups, after an i.p. injection of hexobarbital (150 mg/kg of body weight). Recovery from unconsciousness was indicated by the full restoration of the righting response, defined as the ability of the animal when placed on its back on a flat surface to snap over its paws three times within 15 s (Shapiro et al., 1989).

**Statistics.** Data were subjected to analysis of variance, and differences were determined with t statistics and the Bonferroni procedure for multiple comparisons.

**Results**

**Growth.** The effects of neonatal treatment with MSG or MK-801 on body weight gain became apparent only after puberty (Fig. 1, top panel). In agreement with previous reports (Shapiro et al., 1989; Veneroni et al., 1990), neonatal exposure to MK-801 alone or 2 mg of MSG alone resulted in a persistent 10 to 15% decrease (P < 0.05) at almost all

**Western Blots.** Hepatic microsomes were isolated as previously described (Agrawal et al., 1991) and assayed by Western blotting for the presence of CYP2C11 and CYP3A2 proteins (Pampori et al., 1995). Microsomal protein (10 μg) was electrophoresed on 0.75-mm-thick SDS-polyacrylamide gels containing 7.5% acrylamide and electroblotted onto nitrocellulose filters. The blots were probed with monoclonal anti-rat CYP2C11 (Oxford Biomedical Research Inc., Oxford MI) and anti-rat CYP3A2 (BD Gentest, Woburn, MA).

The primary antibody was located with horseradish peroxidase conjugated to anti-mouse or anti-goat IgG and detected with an enhanced chemiluminescence kit (Amersham Biosciences, Des Plaines, IL). Quantification of relative P450 levels was done by laser densitometry of the X-ray films, normalized to a positive control run on every blot.

**RNA Analysis.** Total hepatic RNA was isolated by using a single-step guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA (10 μg) was electrophoresed under formaldehyde-denaturing conditions on 1% agarose and transcribed to GeneScreen nylon membranes (PerkinElmer Life Sciences, Boston, MA). The Northern blots were probed and reprobed with 32P-labeled oligonucleotide probes, using hybridization and high stringency washing conditions as described previously (Waxman, 1991). The nucleotide sequence of oligonucleotide probes for CYP2C11 (Waxman, 1991) and CYP3A2 (Ram and Waxman, 1991) have been reported. The consistency of RNA loading between samples was confirmed by ethidium bromide staining of 18S and 28S ribosomal RNAs and was verified using an 18S oligonucleotide probe (Rumshen et al., 1993). The hybridized mRNA signals were quantified by scanning the autoradiographs and normalized to the 18S RNA signals in each lane.

**Growth Hormone.** Eight-hour plasma growth hormone profiles were determined by using a homologous radioimmunoassay with a sensitivity of 2 to 3 ng/ml. All values were normalized by subtracting values obtained from hypophysectomized rats. Procedural details and statistical validation of the assay have been reported by us elsewhere (Shapiro et al., 1989; Agrawal et al., 1991).
postpubertal ages) in body weight, whereas the higher dose of the food additive (4 mg of MSG) produced a long-lasting ~30% decrease in body weight \((P < 0.01)\) compared with the vehicle treatment group.\(^1\) Because MK-801 alone had as similar a depressive effect on body weight gain as 2 mg of MSG alone, it is not surprising that the combined treatment resulted in no protective effect by the NMDA receptor antagonist on body weight (Fig. 1, bottom panel). In contrast, concurrent administration of MK-801 completely prevented the deleterious effects of the 4-mg MSG dose on subsequent body weight gain.

The effects of neonatal exposure to MSG or MK-801 on tail length, a measure of skeletal growth, was clearly expressed before or very early in puberty (Fig. 2, top panel). Neonatal administration of MK-801 alone or 2 mg of MSG alone produced a similar ~10% decrease \((P < 0.05)\) in tail length, whereas treatment with the 4-mg dose of MSG resulted in a persistent ~35% reduction \((P < 0.01)\) in tail length throughout the 4-month study period. Having similar effects when administered separately, there was expectedly no combined effect \((P > 0.05\) at almost all ages) of MK-801 and 2 mg of MSG on tail length (Fig. 2, bottom panel). In contrast, concurrent administration of the NMDA receptor antagonist dramatically reduced \((P < 0.01\), although not quite to normal) the permanent effects of the 4-mg dose of MSG on tail growth.

Although tail length is an indicator of skeletal growth, the Lee index is a measure of obesity (Shapiro et al., 1989). In this regard, whereas neonatal treatment with MK-801 induced a delayed but small, persistent decrease in both body weight gain and tail length, the changes were proportional because there was no evidence of obesity (Table 1). In contrast, MSG administration induced a long-lasting, dose-dependent obesity that was hardly noticeable at the 2-mg dose but was startlingly apparent in the 4-mg-treated male rats. Neonatal administration of MK-801 prevented this MSG-induced obesity. Likewise, concurrent administration of MK-801 with either dose of MSG prevented the subnormal kidney, pituitary, and seminal vesicle weights observed in adults neonatally exposed to the amino acid alone (Table 1).

\(^{1}\) Irrespective of the measured endpoint, the effects of the NaCl vehicle treatment and phosphate-buffered saline vehicle treatment were indistinguishable so that all results from both groups were combined into a single "vehicle" treatment group.
Drug Metabolism and Hepatic P450 Levels. Hexobarbital-induced sleeping time, a functional measure of drug action, is directly dependent on but inversely related to hepatic microsomal hexobarbital hydroxylase activity (Pampori et al., 1991b). We found that neonatal exposure to either MK-801 alone or 2 mg of MSG alone had no effect on hexobarbital-induced sleeping time when determined in adulthood (Table 1). In contrast, neonatal administration of the higher 4-mg dose of MSG resulted in adult sleep times that were twice as long as those observed in control rats. However, concurrent administration of MK-801 completely prevented this effect of 4 mg of MSG.

Prolonged sleeping times are undoubtedly due to a commensurate decline in hexobarbital hydroxylase activity (Shapiro and Szczotka, 1984; Pampori et al., 1991b). Since microsomal hexobarbital hydroxylase represents the contributing activities of several isoforms of P450, of which CYP2C11 and CYP3A2 are dominant (Ryan and Levin, 1990), we determined the expression levels of these male-specific P450s in our animals. As previously reported (Shapiro et al., 1989; Pampori and Shapiro, 2000) neonatal administration of 2 mg of MSG/g of body weight produces a permanent, albeit small (~20%), over-expression of CYP2C11 protein that was not corrected by coadministration of MK-801 (Fig. 3). Neonatal treatment with 4 mg of MSG virtually eliminated CYP2C11 protein levels in treated rats. Concurrent treatment with MK-801 not only prevented the suppression of CYP2C11 in male rats treated with 4 mg of MSG, but also induced a slight but persistent over-expression of the isoform.

Adult levels of CYP3A2, another male-specific isoform, were unaffected by neonatal exposure to MK-801 or 2 mg of MSG. Like CYP2C11, neonatal administration nearly eliminated all traces of CYP3A2 protein in adult liver. Co-administration of the NMDA receptor antagonist was fully effective in blocking the suppressive effects of 4 mg of MSG on adult CYP3A2 protein (Fig. 3).

In the absence of substantial effects of the 2-mg dose of MSG on CYP2C11 and CYP3A2 protein levels, we limited mRNA measurements to rats exposed to the higher 4-mg dose of MSG. In this regard, mRNA levels were very much in agreement with protein findings. Whereas neonatal administration of MK-801 alone had no effect on CYP2C11 mRNA levels at 6 months of age, exposure to 4 mg of MSG nearly eliminated adult expression of the isoform (Fig. 4). Concurrent administration of MK-801 to the newborns not only prevented the suppressive effects of 4 mg of MSG given neonatally but, like the protein levels, also caused a small over-expression of the transcript in adulthood.

Similarly, neonatal treatment with MK-801 alone had no effect on adult levels of CYP3A2 mRNA (Fig. 4). Whereas adult concentrations of CYP3A2 mRNA were fully suppressed by neonatal treatment with 4 mg of MSG, coadministration of MK-801 completely blocked the suppressive effects of the amino acid on expression levels of the transcript.

**TABLE 1**

<table>
<thead>
<tr>
<th>Neonatal Treatment</th>
<th>Body Weight</th>
<th>Lee Index</th>
<th>Kidneys</th>
<th>Seminal Vesicles</th>
<th>Pituitary</th>
<th>Sleeping Times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100 g b.wt.</td>
<td>mg/g b.wt.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>622 ± 41b</td>
<td>274 ± 20c</td>
<td>841 ± 10</td>
<td>648 ± 71</td>
<td>98 ± 17</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>2 mg MSG</td>
<td>521 ± 27a</td>
<td>241 ± 20c</td>
<td>411 ± 27</td>
<td>675 ± 56</td>
<td>108 ± 17</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>4 mg MSG</td>
<td>529 ± 29b</td>
<td>338 ± 18c</td>
<td>438 ± 5</td>
<td>540 ± 45</td>
<td>88 ± 13</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>MK-801</td>
<td>557 ± 99b</td>
<td>324 ± 20c</td>
<td>453 ± 21</td>
<td>659 ± 46</td>
<td>98 ± 27</td>
<td>2.9 ± 0.5†</td>
</tr>
<tr>
<td>2 mg MSG + MK-801</td>
<td>557 ± 99b</td>
<td>324 ± 20c</td>
<td>453 ± 21</td>
<td>659 ± 46</td>
<td>98 ± 27</td>
<td>2.9 ± 0.5†</td>
</tr>
<tr>
<td>4 mg MSG + MK-801</td>
<td>557 ± 99b</td>
<td>324 ± 20c</td>
<td>453 ± 21</td>
<td>659 ± 46</td>
<td>98 ± 27</td>
<td>2.9 ± 0.5†</td>
</tr>
</tbody>
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*P < 0.05 compared to rats treated with the same dose of MSG.
†P < 0.05, ††P < 0.01 compared to rats treated with the same dose of MSG.

![Fig. 3.](image) Hepatic CYP2C11 and CYP3A2 protein levels in 6-month-old male rats neonatally treated with MSG and/or MK-801. Pups were injected with either 2 or 4 mg of MSG/g of body weight and/or 1 μg of MK-801/g of body weight or an equivalent volume of vehicle on days 1, 3, 5, 7, and 9 of life. Each data point is a mean ± S.D. with n = 6 rats/group.

![Fig. 4.](image) Hepatic CYP2C11 and CYP3A2 protein levels in 6-month-old male rats neonatally treated with MSG and/or MK-801. Pups were injected with either 2 or 4 mg of MSG/g of body weight and/or 1 μg of MK-801/g of body weight or an equivalent volume of vehicle on days 1, 3, 5, 7, and 9 of life. Each data point is a mean ± S.D. with n = 6 rats/group.
Because expression levels of both CYP2C11 and CYP3A2 are profoundly regulated by sex-dependent signals in the circulating profiles of growth hormone (Agrawal and Shapiro, 2000, 2001), we examined this relationship in adult rats exposed as neonates to MSG and/or MK-801. The established masculine growth hormone profile (Shapiro et al., 1989; Pampori et al., 1991a) was found in our adult vehicle-treated male rats (Fig. 5). Generally, growth hormone was released in pulses approximately every 3 to 4 h, resulting in short-lived peaks of 200 to 250 ng/ml of plasma, followed by ~2.5 h of undetectable (<2–3 ng/ml) trough levels. Neonatal treatment with MK-801 resulted in no alterations in the masculine profile in adulthood. Although there were basically no measurable levels of growth hormone in any of the plasma samples obtained during 8 continuous h of serial blood collections from adult males treated neonatally with 4 mg of MSG, concurrent administration of MK-801 completely abolished the deleterious effects of the amino acid, resulting in normal circulating masculine growth hormone profiles in adulthood (Fig. 5).

**Discussion**

MK-801 is a highly potent and selective noncompetitive NMDA receptor antagonist (Wong et al., 1988) known to prevent the neurodegeneration normally induced by cerebral asphyxia (Ford et al., 1989) and glutamate toxicity (Lehmann and Jonsson, 1992) in neonatal rats and mice. In the present report, the long-lasting deleterious effects of neonatal exposure to MSG were effectively blocked by concurrent administration of MK-801. That is, neonatal MSG (4 mg/g of body weight) produced a persistent suppression in body weight gain, linear growth, and measured organ weights; a notable obesity; a doubling of hexobarbital-induced sleeping times; and repression of hepatic CYP2C11 and CYP3A2 expression as well as suppression of plasma growth hormone secretion to barely detectable levels. All of these effects were prevented by MK-801, which supports the contention that the adult suppression of P450s by neonatal MSG can be explained by the permanent absence of growth hormone from the circulation. However, if the suppression of male-specific CYP2C11 and CYP3A2 in MSG-treated rats is simply due to an absence of the masculine plasma growth hormone profile (and its intrinsic inductive signals), it is unclear why the renaturalized physiologic profile was far from effective in restoring male-like levels of the isoforms. In comparison, restoration of

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**Fig. 4.** Hepatic CYP2C11 and CYP3A2 mRNA levels in 6-month-old male rats neonatally treated with MSG and/or MK-801. Pups were injected with 4 mg of MSG/g of body weight and/or 1 μg of MK-801/g of body weight or an equivalent volume of vehicle on days 1, 3, 5, 7, and 9 of life. Each data point is a mean ± S.D. with n ≥ 6 and expressed as a percentage of the mean value of the vehicle-treated control here designated as 100%. *, P < 0.01; compared with the vehicle control.

**Fig. 5.** Circulating levels of plasma growth hormone in male rats treated neonatally with MSG and/or MK-801. Pups were injected with either 4 mg of MSG/g of body weight and/or 1 μg of MK-801/g of body weight or an equivalent volume of vehicle on days 1, 3, 5, 7, and 9 of life. Plasma was obtained from 6- to 7-month-old individual, undisturbed, catheterized rats at 15-min intervals for 8 continuous h. Similar results were obtained from two to three additional animals in each treatment group.
the same masculine growth hormone profile to another model of growth hormone depletion, the hypophysectomized rat, was completely effective in restoring male-like levels of CYP2C11 and CYP3A2 (Shapiro et al., 1993; Waxman et al., 1995).

This apparent inconsistency in the different response of hepatic CYP2C11 and CYP3A2 to growth hormone replacement in hypophysectomized and MSG-treated rats may be related to the fact that the former experiences a limited, postsurgical period of growth hormone depletion, whereas the neonatally MSG-treated rat lacks growth hormone for its entire life, including the critical period of differentiation of the hypothalamic-pituitary-hepatic axis (Gustafsson et al., 1977). This early growth hormone deficiency may interfere with the development of the growth hormone receptor and/or the signal transduction mechanism(s) that normally mediate growth hormone regulation of P450 expression (Waxman and Frank, 2000), which in turn could result in a permanent insensitivity of the liver to normal growth hormone secretory profiles.

Alternatively, MSG-treated rats may not be totally devoid of growth hormone but may actually secrete hormone levels that are too low for detection by radioimmunoassay. Unlike the hypophysectomized rat, the MSG-treated rat has a pituitary, albeit containing greatly reduced levels of growth hormone (Shapiro et al., 1986). Although MSG-induced lesions in the arcuate nucleus profoundly inhibit secretion of growth hormone-releasing factor (Millard et al., 1982; Bloch et al., 1984), a failure of the pituitary to respond to growth hormone-releasing factor, the pituitaries of affected rats are responsive to growth hormone-releasing factor (Dhir et al., 2002), and it is possible that low, continuous levels of hormone “leak” from the pituitary. We have found that plasma from MSG-treated rats consistently displaces a very small amount of radioactive growth hormone ligand from its specific antibody in our radioimmunoassay (N. A. Pampori and B. H. Shapiro, unpublished data), suggesting the possible presence of very low plasma levels of hormone in these rats. Unfortunately, the displacement is so small that it extrapolates below the sensitivity of the assay (<3 ng/ml) and cannot be statistically validated. Accordingly, restoration of a feminine profile of continuous growth hormone secretion at subdetectable levels, i.e., 1 to 2 ng/ml of plasma, can substantially suppress expression levels of CYP2C11 and CYP3A2 in male rats (Pampori and Shapiro, 1999). Moreover, the suppressive effect of these subdetectable levels of a continuous hormone profile are sufficient to block the inductive effects of the normal masculine episodic growth hormone profile on CYP2C11 and CYP3A2 expression (Pampori et al., 2001). Thus, it is possible that the presence of very low concentrations of continuously secreted growth hormone in MSG-treated male rats inhibits the inductive effect of the restored masculine profile on CYP2C11 and CYP3A2.

In summary, the present findings indicate that the permanent, but latently expressed, effects in drug metabolism and growth hormone secretion induced by neonatal administration of MSG are solely a result of neuronal (i.e., hypothalamic) damage produced through the NMDA receptor at the time of MSG exposure. The irreversibility of the damage, however, may be due to secondary defects resulting from the initial neuronal lesions; i.e., abnormal imprinting of the developing hypothalamic-pituitary-hepatic axis regulating P450 expression and/or pituitary secretion of continuous, but subdetectable, growth hormone levels.

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