EFFECTS OF METHADONE HYDROCHLORIDE ON THE GROWTH OF ORGANOTYPIC CEREBELLAR CULTURES PREPARED FROM METHADONE-TOLERANT AND CONTROL RATS


Department of Pathology, College of Physicians and Surgeons of Columbia University, Department of Neuropathology and Neurotoxicology, New York State Psychiatric Institute and Bronx Psychiatric Center and the Brain Tissue Culture Laboratory, New York State Research Institute for Mental Retardation, New York, New York

Accepted for publication June 4, 1976

ABSTRACT


Male and female Sprague-Dawley rats were given d,l-methadone (5 mg/kg) for at least 3 months and then mated. The drug was continued throughout pregnancy and after delivery. The newly born pups were divided into two groups. One group was tested for in vivo methadone tolerance, while the animals in the other group were used to prepare organotypic cerebellar cultures. Various amounts of d,l-methadone were added to the media of half of these cerebellum cultures. The effect of the drug in the medium was assessed by measuring explant outgrowth. Similar experiments were carried out with control animals. Statistical analysis of the data obtained in the in vivo portion of the experiment indicates that the pups of methadone-treated mothers tolerate methadone better than those of untreated mothers. The culture experiments revealed that the addition of methadone to the medium reduced explant outgrowth size and this was a dose-related effect. Also, there was significantly less outgrowth from explants prepared using pups of methadone-treated mothers as compared to the controls. There was no significant difference in the effect of methadone on the growth of cultures prepared from the methadone-tolerant and control animals.

Methadone hydrochloride is a synthetic narcotic with multiple actions the most prominent of which involve the central nervous system and organs containing smooth muscle. Although the analgesic and sedative properties of narcotics usually receive most emphasis, cell culture studies have shown that some are capable of retarding the growth of immature nervous system tissues (Ghadarian, 1969). Methadone crosses the placental barrier and accumulates in measurable amounts in fetal tissues, including brain (Peters et al., 1972). Although abnormalities have been reported in the offspring of methadone-treated animals (Buchenauer et al., 1974), they are not of the magnitude that would be expected if in vivo drug activity paralleled...
that in vitro. There are a number of plausible explanations for the greater in vitro toxicity of the drug, including the possibility that individual fetal cells develop tolerance to the growth retardant action of methadone.

Tolerance may develop following the administration of a wide variety of agents and it has been shown that tolerance to narcotics can develop in utero (Johannesson and Becker, 1972). Tolerance sometimes appears after a single exposure but is generally most obvious clinically when an agent has been administered chronically. Its manifestations are quite variable as are the means by which it is measured (Cochin, 1970). Some investigators (Richter and Goldstein, 1970; Kalant et al., 1971; Misra et al., 1973) have suggested that tolerance may be due to decreased sensitivity of individual cells because of prolonged occupation of specific cell receptor sites by agents which blockade the receptors or modify them biochemically. Others have postulated that altered drug metabolism (Sung et al., 1953; Olsen, 1972) particularly in the liver (Sung and Way, 1950), could be a factor. Another interesting possibility is that neutralizing antibodies (Kornetsky and Cochin, 1964; Liu and Adler, 1973) play a role.

The present study was designed to investigate the possibility that individual central nervous cells develop tolerance to the growth-inhibiting action of methadone in utero. To do this, cultured cerebellar cells from methadone-tolerant and control pups were maintained in a methadone-containing culture medium. The pharmacologic activity of the drug was assessed by measuring its influence on explant outgrowth. The use of organotypic cell cultures eliminates many of the systemic factors which complicate in vivo studies.

**Methods and Materials**

Young (100 g) male and female Sprague-Dawley rats were given daily subcutaneous injections of methadone hydrochloride (Dolophine hydrochloride, Eli Lilly and Company, Indianapolis, Ind.) (4,4-diphenyl-6 dimethylamino-heptanone-3 hydrochloride). The initial dose (2 mg/kg) was increased by increments (0.5 mg/kg) every 7 days until a maintenance level (5 mg/kg) was reached. The animals were kept at this dosage for 2 months and then mated. Methadone (5 mg/kg) was administered throughout pregnancy and on the day of delivery.

Each litter of newborns was randomly divided into two groups. The pups of the first group (M1) were used for in vivo methadone tolerance tests. These animals were given a single subcutaneous injection of methadone (5-15 mg/kg) 12 to 24 hours after birth. The number of animals alive 6 hours later was recorded. Organotypic cerebellar cultures were prepared from 12 to 24 and 48 to 72 hours old pups of the second group (M2) using standard techniques (Bornstein and Murray, 1958). Following removal of the meninges, each cerebellum was dissected into 10 approximately equal pieces. The tapering end segments were discarded and the remaining eight fragments were explanted on collagen-coated coverslips mounted in a no. 1006 Falcon plastic Petri dish. Control explants were maintained in a solution composed of heat inactivated fetal calf serum (35%), Eagles minimum essential medium (42%), Simm’s balanced salt solution (20%) and 20% glucose (3).

Methadone, concentration 1 × 10⁻³ M in the total solution, was added to the medium of half of the cultures prepared from each litter of 12 to 24 hours old animals. Equal numbers of randomly selected explants from 48 to 72 hours old pups were maintained in the following solutions: 1) standard medium; 2) standard medium + methadone (10⁻⁴ M); 3) standard medium + methadone (10⁻³ M); 4) standard medium + methadone (10⁻² M). All explants were kept in an incubator under the following conditions: atmosphere 95% air, 5% CO₂; temperature 35°C; relative humidity 98%; (Schneider and Rue, 1974).

The cultures were examined after 48 hours. Those that were excessively granular or from which the meninges had not been completely removed were discarded. The outgrowth zone of the remaining cultures was measured using a Zeiss net micrometer viewed at magnification 125×. The original explant was still well delineated at 48 hours so it was possible to measure the size of the explant and the outgrowth zone separately. The extent of the outgrowth, expressed in terms of the number of micrometer squares (1 square µ = 50 µ²) which it covered, was determined and an average outgrowth area size was calculated for each litter subgroup (approximately 30 cultures).

A group of control Sprague-Dawley rats, siblings of those given methadone, were bred at approximately the same age as the treated animals. The progeny of these matings were randomly separated into litter groups (C₁, C₂) and the animals and/or cultures prepared from them were tested in the same way as those in groups M₁ and M₂.

**Statistical evaluation.** The data from the in vivo experiment were analyzed using the Mantel-Haenszel chi-square test for data combined from different 2 × 2 tables (Fleiss, 1973). A split-plot analysis of variance (Snedecor and Cochran, 1967) was used to examine the data from the portion of the in vitro experiment involving cultures prepared from 12 to 24 hours old.
Mortality of rat pups 6 hours after the subcutaneous injection of methadone hydrochloride (5-15 mg/kg)

M1, pups of methadone-treated mothers; C1, pups of control mothers.

<table>
<thead>
<tr>
<th>Methadone mg/kg</th>
<th>Group M1</th>
<th></th>
<th>Group C1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals</td>
<td>Deaths</td>
<td>% Dying</td>
<td>No. of animals</td>
</tr>
<tr>
<td>5.0</td>
<td>30</td>
<td>1</td>
<td>3%</td>
<td>24</td>
</tr>
<tr>
<td>7.5</td>
<td>25</td>
<td>2</td>
<td>8%</td>
<td>29</td>
</tr>
<tr>
<td>10.0</td>
<td>25</td>
<td>4</td>
<td>16%</td>
<td>33</td>
</tr>
<tr>
<td>12.5</td>
<td>59</td>
<td>28</td>
<td>47%</td>
<td>41</td>
</tr>
<tr>
<td>15.0</td>
<td>26</td>
<td>17</td>
<td>65%</td>
<td>41</td>
</tr>
</tbody>
</table>

Effect of methadone hydrochloride (1 x 10^-4 M) in culture medium on explant outgrowth size

Values are mean of at least 300 cultures. M2, cultures prepared from 12 to 24 hours old methadone-tolerant animals; C2, cultures prepared from 12 to 24 hours old control animals; m, methadone in culture medium (concentration 1 x 10^-4 M); s, standard medium.

<table>
<thead>
<tr>
<th>Litter No.</th>
<th>Group M2</th>
<th></th>
<th>Group C2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>m</td>
<td>s</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>70</td>
<td>26</td>
<td>140</td>
<td>86</td>
</tr>
<tr>
<td>2</td>
<td>111</td>
<td>42</td>
<td>160</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>51</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>121</td>
<td>51</td>
<td>154</td>
<td>102</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>47</td>
<td>142</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>48</td>
<td>121</td>
<td>65</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>75</td>
<td>163</td>
<td>122</td>
</tr>
<tr>
<td>8</td>
<td>120</td>
<td>45</td>
<td>148</td>
<td>102</td>
</tr>
<tr>
<td>9</td>
<td>151</td>
<td>100</td>
<td>113</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>101</td>
<td>52</td>
<td>148</td>
<td>96</td>
</tr>
<tr>
<td>11</td>
<td>117</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>103.2</td>
<td>52.3</td>
<td>143.9</td>
<td>83.8</td>
</tr>
<tr>
<td>S.D.</td>
<td>24.8</td>
<td>19.7</td>
<td>16.0</td>
<td>23.2</td>
</tr>
</tbody>
</table>

*1 micrometer square = 50 µ².

Results

Nearly all of the animals tested in vivo showed evidence of a drug effect within several minutes after receiving methadone. The earliest sign was decreased body movement followed by cyanosis and a marked fall in respiratory rate. Most of the animals that died did so within the 1st hour following injection. Many animals, particularly those receiving small amounts of the drug, were reviving by the end of the 6-hour observation period. The results of the in vivo tests are summarized in table 1. Analysis of these data revealed statistically significant differences in the mortality rates of groups M1 and C1 (χ² = 11.42, df = 1, P < .01). This means that the animals in group M1 tolerated methadone better than the animals in group C1.

The results of the in vitro studies of the effect of methadone on the outgrowth of cerebellar cultures prepared from 12 to 24 hours old pups are presented in table 2. The average size of the outgrowths from explants prepared from the offspring of methadone-treated mothers (M2m, M2m) was significantly smaller than the outgrowth of the controls (C2m, C2m); the average difference between the outgrowths in groups M2 and C2 was 36.1 squares (t = 4.46, P < .01). The difference between groups C2 and M2m was 40.7 squares (t = 4.42, P < .01) and between C2m and M2m, 31.5 squares (t = 3.36, P < .01).

The addition of methadone to the culture medium significantly inhibited outgrowth size. The average difference between the methadone treated cultures (C2m, M2m) and the untreated cultures (C2m, M2m) was 55.5 squares (t = 12.19, P < .01).

The addition of methadone to the culture medium had essentially the same effect on cultures prepared from the offspring of methadone-treated mothers (M2) as it did on the controls (C2). The mean difference in culture outgrowth size between groups M2 and M2m,
50.9 squares was not significantly different from the mean difference between groups $C_{25}$ and $C_{2m}$ 60.1 squares ($t = 1.01$, N.S.).

The data showing the effect that different methadone concentrations in the culture medium had on the growth of explants from 48 to 72 hours old animals are summarized in table 3. The mean growth of the pretreated group cultures maintained in the standard medium ($M_{25}$) was slightly less than the mean growth in the control group ($C_{25}$), but not significantly so ($t = 1.08$, N.S.).

Figure 1 presents the least-squares lines associating outgrowth size with log dose of methadone for the two groups. The slopes of the lines, $-38.6$ micrometer squares per 10-fold increase in dose for the pretreated group ($M_2$) and $-29.9$ for the control group ($C_2$) were not significantly different ($t = 1.17$, N.S.), which suggests that the lines might be parallel. The mean slope, $-34.2$ micrometer squares per 10-fold increase in dose, was significantly different from $0$ ($t = 9.21$, $P < .01$). The two lines were, on the average, separated by $39.4$ micrometer squares ($t = 3.13$, $P < .01$). Growth with methadone in the culture medium was therefore significantly less for the explants from the pretreated group ($M_2$) than for those from the control group ($C_2$).

To examine the phenomenon of growth suppression, each outgrowth size value associated with the presence of methadone in the culture medium was subtracted from the corresponding value for the standard medium and the resulting values were associated with log dose of methadone (fig. 2). The slopes of the lines are algebraically equal to those in figure 1, but the lines are not significantly displaced, the mean difference being $19.1$ micrometer squares ($t = 0.98$, N.S.). Suppression of growth therefore seems to be the same in the pretreated as in the control group.

**Discussion**

Previous *in vitro* studies have yielded conflicting data concerning the ability of cultured cells to develop tolerance to narcotics (Painter *et al.*, 1949; Corsen and Skora, 1964; Ruffin *et al.*, 1969; Liss, 1972). This subject was reviewed by Grode and Murray (1973) who tried unsuccessfully to induce tolerance in organotypic dorsal root ganglia cultures by exposing them to methadone for periods of up to 55 days. In our experiment, freshly cultured central nervous system cells from presumably methadone-tolerant animals were tested for tolerance, using cell growth suppression as an index. It should be pointed out that the growth retardation which we observed is probably not a specific narcotic effect since cerebellar tissues are believed to have few, if any, opiate-binding sites (Pert and Snyder, 1973). This does not mean, however, that tolerance to cell growth suppression induced by an opiate cannot develop. In fact, there is evidence from other studies employing cultured cerebellar tissues that it does occur (Ghadirian, 1969).

The observation that the growth of cultures prepared from the offspring of methadone-treated mothers was slowed is of interest. It seems likely that this was caused by the presence of methadone in the freshly explanted tissues, although the persistence of a drug-induced cell depression not related to continued drug presence cannot be ruled out. If the action of methadone in intact animals paralleled that in the cultures, grossly detectable abnormalities of cerebellar development would probably be present in the pups born to

**TABLE 3**

*Effect of various concentrations of methadone hydrochloride in culture medium on explant outgrowth size*

<table>
<thead>
<tr>
<th></th>
<th>Group $M_2$</th>
<th>Group $C_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>Mean outgrowth (micrometer squares)</td>
<td>205.3</td>
<td>155.5</td>
</tr>
<tr>
<td>S.D.</td>
<td>46.1</td>
<td>30.0</td>
</tr>
</tbody>
</table>

*Concentration of methadone (moles/liter).*

*1 micrometer square = 50 $\mu^2$.*
Fig. 1. Effect on explant outgrowth produced by the addition of methadone to the media of cultures prepared from methadone-tolerant (M₂) and control (C₂) animals. Each point along the lines represents the mean outgrowth size of approximately 300 cultures.

Fig. 2. Suppression by methadone of the outgrowth from explants prepared using methadone-tolerant (M₂) and control (C₂) animals. The mean outgrowth sizes of the cultures grown in various methadone-containing media were subtracted from those of the explants maintained in the standard medium and the differences related to medium methadone concentration.

methadone-treated mothers. However, no significant macroscopic dissimilarities were observed between the cerebellums of the animals in the latter group and the controls. This suggests that there are differences in the in vivo and in vitro activity of methadone.

The sizes of the outgrowth areas in both the pretreated and control groups were greater for
if more tissue is used. Another interesting finding was that methadone had the same effect on the growth of cell cultures prepared from tolerant and control animals. In other words, at least in regard to the growth-retardant effect of methadone, the isolated cerebellar cells from tolerant animals were not tolerant. This does not mean, however, that tissues elsewhere in the nervous system (i.e., basal ganglia, hippocampus, etc.) must react in a similar way. It is also important to realize that the culture test system is not identical to the in vivo situation. Drug-cell relationships which exist in intact animals may differ from those in this experiment, or tolerance may be dependent on the action of a methadone metabolite (Misra et al., 1972; Sullivan et al., 1972) which is not present in the cultures. Finally, the fact that the newborn pups showed tolerance to lethality does not mean that their central nervous system cells have to show tolerance to the growth-inhibiting properties of methadone.

In conclusion, the results of this experiment support previous studies indicating that drug tolerance can develop in very young animals, even those exposed in utero. The observation that in vitro growth of central nervous system tissues is inhibited by methadone raises serious questions concerning the advisability of chronic methadone use during pregnancy, even in a therapeutical setting. We did not observe gross methadone-related cerebellar abnormalities but the possibility that brain damage, not detectable at a macroscopic level, occurs deserves further investigation. Finally, the results of this study do not support the hypothesis that individual fetal central nervous system cells develop tolerance to the growth suppressant action of methadone in utero.

Acknowledgments. The authors wish to acknowledge the assistance provided by Mrs. Margaret Van Cleef, Mr. Elio Perez and Mr. Steven Bodansky.

References


