0031-3998/07/6205-0533
PEDIATRIC RESEARCH
Vol. 62, No. 5, 2007
Printed in U.S.A.

Inhibition of Milk Ingestion and Growth After Administration of a Neutral Cannabinoid CB_1 Receptor Antagonist on the First Postnatal Day in the Mouse

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ABSTRACT: We have shown previously that neonatal exposure to the cannabinoid CB1 receptor antagonist/inverse agonist rimonabant (SR141716) interfered with suckling and development. However, it was not clear whether the developmental deficiencies were induced by neutral CB1 receptor blockade, thereby inhibiting endogenous cannabinoid "tone," or by inverse agonist reduction of constitutive CB1 receptors. CB1 receptor blockade supports our hypothesis that low CB1 receptor concentrations and/or reduced endocannabinoid levels underlie infant nonorganic failure to thrive (NOFTT). Inverse agonism implies that lower constitutive CB1 receptor activity may be responsible for impaired food intake in newborns. In the present study, we injected the neutral CB1 receptor antagonist 5-(4-chlorophenyl)-3-[(E)-2-cyclohexylethenyl]-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole (VCHSR1) to 1-d-old mouse pups and recorded weight gain, gastric milk contents (milkbands), axillary temperature, and survival between age 1 and 10 d. The results showed a dose-related interference with all measures. These data show that (1) growth failure induced by rimonabant is generalized to another CB1 antagonist and (2) cannabinoid CB1 receptor activation by endocannabinoids is essential for normal milk ingestion and development in mice. This supports our hypothesis that endocannabinoid deficiency and perhaps CB1 receptor dysfunction represents the uncharacterized biologic vulnerability, which underlies NOFTT. **(*Pediatr Res* 62: 533–536, 2007)**

We have shown previously that administration of the cannabinoid CB1 receptor antagonist/inverse agonist N-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (SR141716 or rimonabant) to newborn mouse pups dramatically interferes with their ability to ingest milk. As a result, these pups display severe growth failure and high mortality rates during the first week of life. These effects are dose dependent and specifically mediated by the cannabinoid CB1 receptor because coadministration of rimonabant with the prototypical cannabinoid receptor agonist Δ (9)-tetrahydrocannabinol (THC) reversed the adverse effects (1,2) and the phenomenon was only partially present in CB1 receptor knockout pups (1). We have also suggested and presented preliminary data that show that the impaired milk ingestion by the rimonabant-treated pups is due to an oral-motor weakness (3,4).

Human infants with growth failure and low rates of food intake, without any organic cause, are classified as suffering from nonorganic failure to thrive (NOFTT). No treatment or biological mechanism to explain this condition has been offered thus far (5). However, an oral-motor weakness is often observed (6–8).

Consequently, we suggested the rimonabant-treated developing mice as a first animal model for NOFTT in infants (4,9,10).

However, rimonabant acts not only as an antagonist (11), but also as an inverse agonist (12,13) and even, at high doses, as an agonist (14). Therefore, in our previous experiments, rimonabant may have been mediating its effects on pup development by an inverse activation of the CB1 receptor, by neutral blockade, or even by high-dose induced activation [in which case THC counteracted rimonabant by a partial agonism-induced inhibition (2,15)]. To better understand the mechanisms underlying our animal model and to extend our observations to a different CB1 receptor antagonist, we have now studied the effects of the neutral (silent) CB1 receptor antagonist 5-(4-chlorophenyl)-3-[(E)-2-cyclohexylethenyl]-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole (VCHSR1) (16,17) on 1-d-old mouse pups.

For rimonabant to be maximally effective, we commonly use a dose of 20 mg/kg (2). VCHSR1 binds with a 14-fold lower affinity (31.3 nM) to the CB1 receptor compared with rimonabant [2.3 nM (16)]. However, its limited solubility permitted a maximal dose of 12.5 mg/kg when administered in the commonly used injection volume of 10 μL/g, but 25 mg/kg when a double volume was used. We therefore injected newborn Sabra mice with 12.5 or 25 mg/kg within 24 h of birth.

Similar to previous studies (1,2), developmental parameters assessed included body weight, milkbands (milk in the stomach is visible through the transparent newborn skin and thus

Received March 6, 2007; accepted June 22, 2007.

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E.F. was supported by an intramural grant from AVCS. H.M. and H.B. were recipients of fellowships for outstanding students granted by AVCS.

Abbreviation: NOFTT, nonorganic failure to thrive

can be conveniently scored), body (axillary) temperature, and mortality between day 0 and 10 of life.

METHODS

Animals. Pregnant mice were purchased from (Harlan, Israel). Birth was monitored at least twice daily *via* an Internet camera. Day 0 of age is defined as within 24 h after birth. For each experiment, four to six litters (Sabra mice give birth to about 10 pups per litter) were injected using a split-litter design with matched littermates: half the pups received VCHSR1 and the other half vehicle of equal injection volume, as described (1,2). Thus, each experimental group was composed of 20–33 pups. Locomotion observations in adult (Sabra) mice were performed in sample sizes of five for each group. This study was approved by the Institutional Review Board of Animal Experimentation.

Injections. For stock solutions, VCHSR1 was dissolved in acetone because solubility was superior over that in ethanol (which is the solvent of choice for *in vivo* experiments with most cannabinoids and also rimonabant [see Fride *et al.* (18)], isopropanol, or dimethyl sulfoxide. Subsequently, cremophor (Sigma Chemical Co., Israel) and, after mixing, saline was added (1:1:18). The maximal solubility in acetone was 13.9 mg/mL. Therefore, when injection volumes were maintained at 10 μL/g per mouse, a dose of 12.5 mg/kg was administered. To achieve a higher (25 mg/kg) dose, injection volumes were increased to 20 μL/mL, whereas control littermates received 20 μL/mL vehicle (acetone:cremophor: saline = 1:1:18).

Procedure. From d 1, pups were assessed daily for body weight; milkbands (milk in the stomach is scored through the transparent skin with a rating scale between 0 and 1 [0–1/4–1/2–3/4–1; see Fride *et al.* (2)] and axillary temperature (TES 1307 K/J thermometer, Bioseb, France). (Milkbands can only be recorded until d 5–6, when fur is starting to develop). During testing, the mother was removed from the cage and kept in a different room. While a litter was examined, the environmental temperature in the cage where the pups were held was maintained at 28°–30°C.

Motor activity in adult mice. Adult (female Sabra) mice were injected intraperitoneally with 10 or 25 mg/kg VCHSR1 or rimonabant (5 mg/kg). Sixty minutes later, locomotion was monitored in an open field (30 × 40 cm, divided into 20 equal-sized squares). Motor activity was assessed by counting the number of squares crossed over a 8-min period (19).

Statistical analyses. Body weights, milkband scores, and axillary temperature data were analyzed by two-way analyses of variance (ANOVAs) with Bonferroni *post hoc* comparisons for group differences on any given day. Survival curves were created with the Kaplan-Meier method, and the curves were compared with a log-rank test (Prism 4, Graphpad). In addition, individual χ^2 tests were performed for each day.

RESULTS

As can be seen from Figure 1, 12.5 mg/kg of VCHSR1 administered on d 1 of life, significantly reduced the milkband score ($F = 9.7$, $df = 1,65$, $p < 0.01$, Fig. 1*A*) and induced persistent axillary hypothermia ($F = 5.9$, $df = 1,78$, $p < 0.02$, Fig. 1*B*). However, these effects did not translate into a significant reduction in body weight gain (Fig. 1*C*). A dose of 25 mg/kg had much greater effects, as can be seen in Figure 2: the VCHSR1-treated pups were significantly impaired in all three measures: milkbands ($F = 8.7$, $df = 1,210$, $p < 0.001$); axillary temperature ($F = 9.9$, $df = 1,200$, $p < 0.002$), and body weight ($F = 16$, $df = 1,294$, $p < 0.0001$). The 25-mg/kg experiment was replicated, and almost exactly the same results were obtained (compare Figures 2 and 3). In adult (Sabra) mice, VCHSR1 (10–25 mg/kg), similarly to rimonabant, did not affect locomotion in an open field (Fig. 4).

Mortality was negligible in pups treated with 12.5 mg/kg (data not shown). However, within a few days of birth, survival was significantly lower in pups treated with 25 mg/kg of VCHSR1 compared with vehicle-treated controls. The difference in survival rates remained significant throughout the first 10 d of life (the period of observation) ($\chi^2 = 3.899$, $df = 1$, $p < 0.05$, Fig. 5).

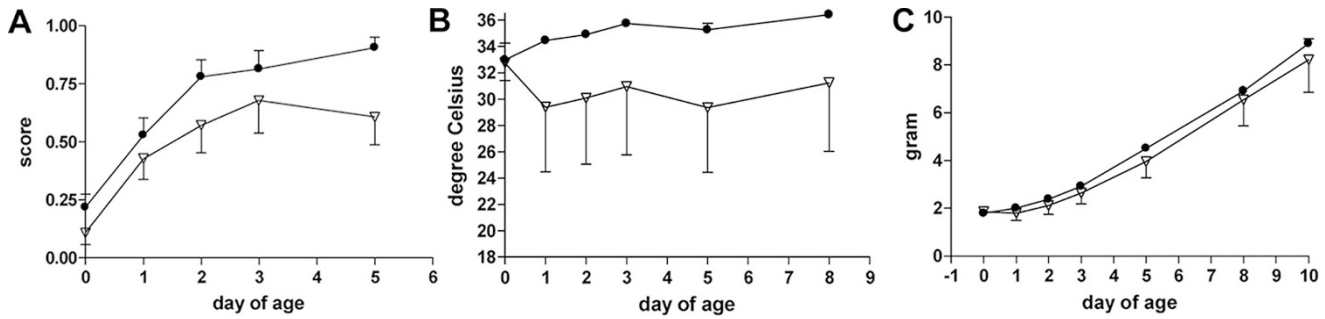

Figure 1. Effects of neonatally injected VCHSR1 (12.5 mg/kg) on postnatal growth and development. After subcutaneous injection of VCHSR1 (▽: 12.5 mg/kg) or vehicle (●) administration on d 0, milkbands (*A*), axillary temperature (*B*), and body weight (*C*) were recorded throughout the first 10 d of life. $n = 20$–22 for each group.

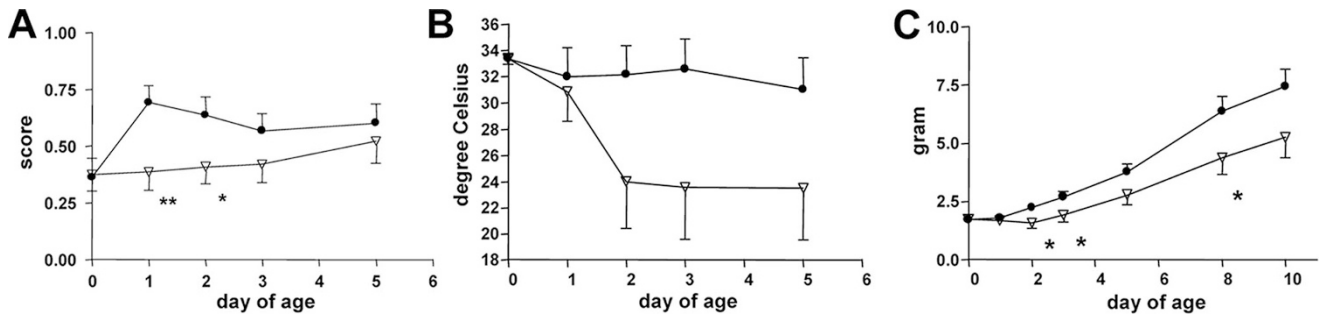

Figure 2. Effects of neonatally injected VCHSR1 (25 mg/kg) on postnatal growth and development. After subcutaneous injection of VCHSR1 (▽: 25 mg/kg) or vehicle (●) administration on d 0, milkbands (*A*), axillary temperature (*B*), and body weight (*C*) were recorded throughout the first 10 d of life. $n = 22$ for each group, $*p < 0.05$ *vs* vehicle; $**p < 0.01$ *vs* vehicle.

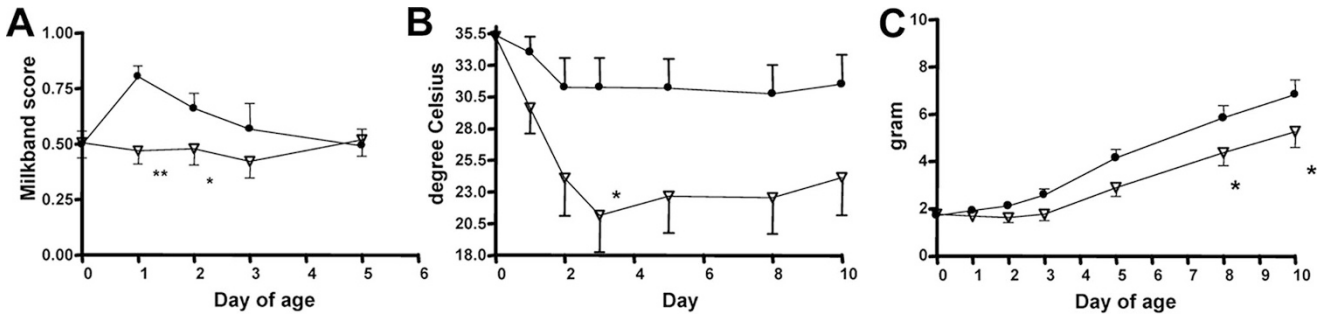

Figure 3. Effects of neonatally injected VCHSR1 (25 mg/kg) on postnatal growth and development (a replication of the experiment presented in Figure 2, performed 6 mo later. VCHSR1 (▽: 25 mg/kg) or vehicle (●). (*A*) milkbands, (*B*) axillary temperature, and (*C*) body weight. Two-way ANOVAs indicated that VCHSR1 induced lower milkband scores ($F = 11.4$, $df = 1,366$, $p < 0.001$), hypothermia ($F = 27.2$, $df = 1,427$, $p < 0.0001$), and weight gain ($F = 16.4$, $df = 1,399$, $p < 0.001$). $n = 26$–33 for each group. *$p < 0.05$ *vs* vehicle; **$p < 0.001$ *vs* vehicle.

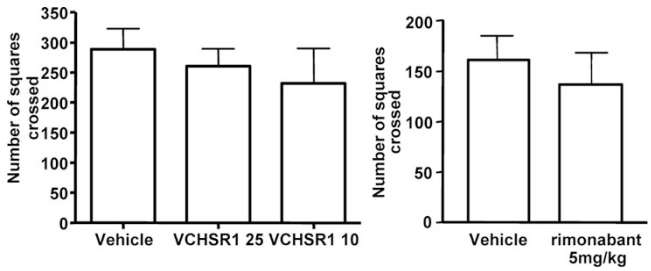

Figure 4. The effect of VCHSR1 on locomotion in adult mice. Adult mice were administered VCHSR1 (10 or 25 mg/kg) or rimonabant (5 mg/kg). Sixty minutes later, locomotion was monitored in an open field (30 × 40 cm, divided into 20 equal-sized squares). Motor activity was assessed by counting the number of squares crossed over a 6-min period.

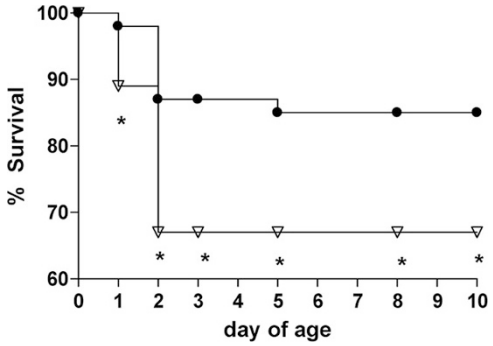

Figure 5. Survival rates in VCHSR1 (▽: 25 mg/kg) or vehicle (●) treated pups. Survival was recorded throughout the first 10 d of life and was recorded as a percentage of the total number of pups born in the respective treatment group. *$p < 0.05$; *vs* vehicle.

DISCUSSION

In the present study, we found that the neutral CB1 receptor antagonist VCHSR1 impaired milk ingestion and postnatal development when administered on d 1 of life in a dose-dependent fashion. Although the effects of VCHSR1 on milkbands (an expression of milk ingestion) and on axillary temperature were statistically significant with both the lower (12.5 mg/kg) and the higher (25 mg/kg) doses, the lower dose effected only a slight depression of the growth curve, whereas a high dose had a highly significant effect. The failure to ingest normal amounts of milk can probably not be ascribed to any effect on motor activity because, at least in adults, VCHSR1 at similar doses as those used for the pups did not affect motor activity. Future studies will analyze suckling and (oral) motor performance in VCHSR1-treated pups. Previous observations on rimonabant had indicated that antagonist-treated pups displayed a selective impairment of nipple attachment (20).

In our earlier observations, using 20 mg/kg of the CB1 receptor antagonist/inverse agonist rimonabant (SR141716), we recorded more dramatic effects including approximately 80% mortality rates, especially in some strains of mice [Sabra and C57BL/6 (1,2)].

However, in view of the much lower binding affinity of VCHSR1 to the CB1 receptor [14-fold lower than that of rimonabant: 2.3 nM *versus* 31.3 nM (16)], it is not surprising that VCHSR1 had a smaller effect on milk ingestion, pup growth, and survival. Rather, the effect observed here is surprisingly large. Therefore, the relatively dramatic effects of VCHSR1 on pup development require an explanation. Brain permeability, as studied for other neutral CB1 receptor antagonists (21), may have played a role in determining the *in vivo* effect. Thus, more effective brain permeability of VCHSR1 could explain the relative effectiveness of VCHSR1 in impairing milk ingestion and pup growth. However, in the absence of such data for VCHSR1, this issue cannot be resolved at present.

Two main conclusions can be drawn from the present study. First, rimonabant-induced effects on pup development and survival, as observed previously, may now be generalized to an additional CB1 receptor inhibitor because VCHSR1 had very similar effects on neonatal feeding and growth to those observed previously with rimonabant (1,2). This supports our hypothesis that CB1 receptor antagonism underlies NOFTT and may serve as a (first) animal model for this enigmatic condition.

Second, the neutral antagonism-induced interference with pup development, as observed here, indicates that endocannabinoid transmission is highly important for milk ingestion in newborn mice. Thus, neutral CB1 receptor blockade rather than inhibition of constitutive CB1 receptor activity (22,23), should be held responsible, at least in part, for the growth-stunting effects of rimonabant observed in our earlier studies (1,2,20). This is consistent with the suppression of feeding observed after administration of the silent antagonist O-2050 (24). However, because a relatively high dose of rimonabant is

required for a severe disruption of milk suckling (1,2), it is possible that when rimonabant was administered, neutral antagonism as well as inverse agonism contributed to the final effect on pup development (13).

Taken together, we conclude that decreased endocannabinoid release interferes with pup food intake, growth, and development. However, because endogenous inverse agonists have been shown to influence constitutive activity, at least in the melanocortin system (22), it is also possible that normal suckling behavior is a function of endocannabinoid release as well as of CB1 receptor constitutive activity.

Therefore, we suggest that NOFTT may be caused by an endocannabinoid deficiency and perhaps additionally by reduced constitutive CB1 receptor activity. Furthermore, we speculate that 2-arachidonoyl glycerol (2-AG) is the pivotal endocannabinoid involved in milk ingestion and pup growth because, first, 2-AG displays a striking (twofold) peak level on the first postnatal day, at least in rat pups (25), and, second, 2-AG was present in maternal milk at high concentrations compared with the presence of only minimal amounts of anandamide (2).

In conclusion, our findings are compatible with a CB1 receptor antagonist-based model for NOFTT. Accordingly, infants with NOFTT are expected to display reduced endocannabinoid (2-AG) release and perhaps impaired CB1 receptor function. Future studies will further investigate this hypothesis.

Acknowledgments. The authors are grateful to Hodaya Dahan for her technical advice and assistance.

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