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Effect of serotonergic anorectics on food intake and induction of Fos in brain of mice with disruption of melanocortin 3 and/or 4 receptors

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ABSTRACT

Previous studies have indicated that type 3 or 4 melanocortin receptors (MCR) are downstream of the critical anorectic action of drugs that stimulate 5-HT_{2C} receptors. To characterize further the receptor types involved, we have studied the effect of serotonergic anorectics in mice with genomic disruption of either MC3R or MC4R, or their combined knockout. In a first experiment, we showed that wild type (WT) and MC4R−/− mice showed comparable inhibition of food intake following acute treatment with dexnorfenfluramine. In a second experiment using WAY-161503, a 5-HT receptor full agonist with selectivity for 2B and 2C subtypes, we found that MC4R−/− responded comparably to WT, while MC3R−/− had reduced sensitivity. Double receptor knockout (DKO) mice responded comparably to WT and MC4R−/−. Surprisingly, brain Fos-ir was not strongly induced in any brain region by WAY-16103 with the exception of the paraventricular nucleus of DKO. These data suggest that MC3Rs may be involved in the response to serotonergic anorectic agents, and more generally in control of food intake.

1. Introduction

Convincing evidence implicates central melanocortin receptors (MCRs) in control of food intake and energy balance (for review, see Adan et al. 2006; Garfield et al., 2009). Many such studies have been in mice with homozygous genetic disruption of either type 3 (MC3R−/−) or type 4 (MC4R−/−) receptors (Chen et al., 2000; Huszar et al., 1997). Pharmacological probes have limited specificity; the most commonly used in behavioral studies has been a mixed type 3/4R agonist, MTII (Hruby et al., 1995), with specific agents under development (Todorovic et al., 2007).

MC4R−/−mice have an obese phenotype that develops in young adulthood, with both males and females reaching adult weights about twice those of their wild type (+/+) counterparts (Huszar et al., 1997). In contrast, MC3R−/− mice are not markedly overweight compared with +/+, but they have been reported to have higher body fat, indicating a shift in energy partitioning (Chen et al., 2000). Because mice are often used as models of obesity, the overwhelming majority of neurobehavioral publications have used MC4R−/−, with very few corresponding studies in MC3R−/−. Interestingly, Chen et al. (2000) also reported that mice bearing a double deletion of both type 3 and 4 receptors (double knockout, DKO) were even more obese than MC4R−/−, suggesting that type 3 and 4 receptors interact in the regulation of energy balance.

Heisler et al. (2002) suggested that the well-known anorectic effects of serotonin (5-HT) agonists were mediated through action on downstream melanocortin pathways. Both rats and mice express 5-HT_{2C} receptors in POMC neurons (Heisler et al., 2002; Xu et al., 2008) and it has been reported that MC4R−/− mice do not show anorexia to chronic administration of a 5HT2c agonist, BTV.X (Lam et al., 2008). Heisler et al. (2006) further reported that nocturnal food intake of juvenile MC4R−/− mice was unaffected by a dose of dexfenfluramine (3 mg/kg) that reduced intake of WT mice by ~50%, whereas MC3R−/− mice showed a similar response to WT. In the present paper we report the effects of a 5-HT releaser and non-selective receptor agonist, dexnorfenfluramine (DNOR) (Rowland and Carlton 1986; Rowland et al., 2000) on food intake in adult MC4R−/− mice and then extend the analysis to a more selective direct 5-HT_{2C} agonist, WAY-161503 (Rosenzweig-Lipson et al., 2006), and to mice with deletion of either type 3 or 4 MCRs, as well as to DKO. We also report the effect of acute injection of WAY-161503 on brain activation, measured using Fos immunoreactivity (Fos-ir).

2. Methods

2.1. Animals and housing

MC3R−/− mice on a mixed C57B6/129 background were generated by breeding heterozygous pairs from a colony originating at Merck Pharmaceutical Co. MC4R−/− mice, also on a 129/B6 background, were generated by breeding heterozygous pairs from a colony originating at Millenium Pharmaceuticals Co. Double knockouts were generated by

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crossing the MC3R−/− and MC4R−/− mice. All mice were genotyped from a tail snip taken at weaning, as described elsewhere (16). Wild type controls were the littermates (+/+) derived from our in house breeding colonies which are maintained in the Cancer Genetics vivarium at the University of Florida. At 2–6 mo of age, mice were moved to the Psychology Department vivarium for test procedures. Both facilities are managed by a centralized and accredited animal care program.

The Psychology vivarium was maintained on a 12:12 light cycle (on: 0700–1900) with an ambient temperature of 23±2 °C and relative humidity 50–70%. For at least a week before and throughout behavioral testing mice were housed singly in standard polycarbonate cages with Sani Chips (Harlan, Madison WI) bedding and with either a compressed paper square (Nestlet) or a red polycarbonate Igloo® (BioServ, Frenchtown NJ) as enrichment (25). Food (#5001 pellets; PMI International, Brentwood MO) and tap water were available at all times, except as noted. Animal use was in accordance with principles in the NRC Guide for Care and Use of Laboratory Animals, and was approved by the University of Florida Institutional Animal Care and Use Committee.

Experimentally-naïve female WT and MC4R−/− mice were used in Experiment 1 at about 3 mo of age. Mice in Experiment 2 had previously served in a study of meal patterns and/or acute feeding tests with CCK, but had at least 1 week of recovery from these tests before any of the presently described procedures were begun. Male and female mice (1–2 male, rest female per group) of DKO, MC4R−/−, MC3R−/− and wild type (+/+) genotypes were used at 5–10 mo of age.

2.2. Behavioral testing

Food intake was measured in 30 min test sessions using a "dessert" protocol in which the mice were adapted to receive a highly preferred food treat at the same time each day. The treat used was Crunchies® (BioServ, Frenchtown NJ), spherical 190 mg pellets of similar macronutrient composition to chow but in three fruit flavors. The choice of this treat avoids any change in macronutrient or water content relative to the maintenance food. During training, mice received 10 pellets of mixed flavors in a 10 ml glass beaker, hung at floor level inside the cage from a metal stirrup. On the first day the beaker was left in place for as long as needed for robust intake to occur. Thereafter, the time per day was tapered rapidly to 30 min. We found that most (>90%) wild type (+/+) mice readily adapted to consume Crunchies whereas a considerable fraction (~25%) of the MC4R−/−, MC3R−/− and DKO mice did not touch this novel but palatable food even after many days of presentation. Sufficient mice were initially trained so that at least 6 reliable eaters were obtained for each genotype. Test intakes were recorded as the number of Crunchies eaten; bedding was searched for half pellets, but these or smaller crumbs were usually minimal. After about 1 week adaptation to the dessert regimen, a baseline was established by recording intake for 3 consecutive days.

2.3. Experiment 1: dexnorfenfluramine

This study used 5 WT (mean±SE body wt 18.2±1.0 g) and 5 MC4R−/− (27.8±1.6 g) female mice. On the first test day, mice were injected subcutaneously at about 1300 h with either the vehicle (saline, .02 ml/10 g body weight) or DNOR (2 mg/kg). Crunchies were presented 15 min later and intake recorded as described above. One week later, the test was repeated with the groups reversed. Another week later, all mice were injected with DNOR (4 mg/kg). The low dose then high dose protocol was chosen to minimize possible development of anorectic tolerance (Rowland et al., 2003). The hydrochloride salt of DNOR was a gift from L'Institut des Recherches Servier. Intakes were expressed as % baseline and were analyzed by ANOVA.

2.4. Experiment 2: WAY-161503

This experiment used male and (mostly) female mice of WT, MC3R−/−, MC4R−/− and DKO verified genotypes. They were tested as above, except that mice received either the saline vehicle or WAY-161503 (4 or 8 mg/kg) in random order. These doses were chosen from our previous work in C57BL/6 mice using this protocol (Rowland et al., 2008). The procedure was repeated at 7 day intervals with each animal receiving each dose, in random order. There was no order effect so the data from all 3 weeks were combined for analysis. Intakes after vehicle or WAY were expressed as % of the baseline for each individual, and the transformed data were analyzed using 1-way ANOVA and post hoc Tukey tests ($P<0.05$). WAY-161503 hydrochloride was purchased from Tocris Biosciences (Ellisville, MO).

After completion of the feeding studies, the same mice were used to study induction of c-Fos immunoreactivity. This was performed as several batches, with all 4 genotypes represented in each batch; no differences were found as a result of batch, so data were combined, as planned. On each test day, chow pellets were removed from the home cages 1–2 h beforehand to prevent recent spontaneous meals. Mice ($N=5$/group) were injected with WAY-161503 (8 mg/kg) and food removed. One mouse from each genotype, plus an extra WT mouse were injected with saline vehicle and formed an 'omnibus control' group. One hour later, mice were anesthetized (Sleepaway, 1 ml/kg; Fort Dodge), and were perfused transcardially with heparinized saline followed by paraformaldehyde. Brains were removed, stored in paraformaldehyde overnight, and then sliced by vibratome into coronal 75 µm sections at the levels of the paraventricular nucleus (PVN) of the hypothalamus and the area postrema (AP) and adjacent nucleus of the tractus solitarius (NTS). Sections were then incubated with primary (c-Fos 4 polyclonal; Santa Cruz) and secondary (biotinylated goat anti-rabbit IgG, Zymed) antibodies, and the reaction product visualized using ABC (Vector Labs), as we have described in detail elsewhere (Li and Rowland 1993). Sections were mounted on slides and c-Fos positive cells in regions of interest were examined under a microscope and counted manually by two observers; to ensure a blind procedure, animal numbers were obscured during this phase. Within an animal and area, the section with the greatest number of positive cells was used as the datum. The counts from the two observers showed <5% variation and were averaged. Counts were compared statistically using 1-way ANOVA and post hoc Tukey tests ($P<0.05$).

3. Results

3.1. Experiment 1: dexnorfenfluramine

Baseline intake of Crunchies did not differ between WT (mean of 7.0 pellets = 1.33 g) and MC4R−/− (6.2 pellets, 1.18 g) groups. The results with DNOR are shown in Fig. 1. DNOR caused a dose related suppression of food intake ($P<0.001$), with no main or interactive effect of genotype. Because the lack of genotype difference might have been dominated by the high dose data, we also ran ANOVA using only the 0 and 2 mg/kg doses. However, once again, the effect of dose was significant ($P=0.012$) but the dose × genotype interaction was not.

3.2. Experiment 2: WAY-161503

The average body weights and baseline intakes of Crunchies of during the 3 study weeks differed significantly as a function of genotype and are shown in Table 1. As expected, both MC4R−/− and DKO weighed more than WT, and MC3R−/− did not differ from WT. The DKO mice weighed less than MC4R−/−, possibly because they were younger. Baseline intake of Crunchies did not differ from WT in

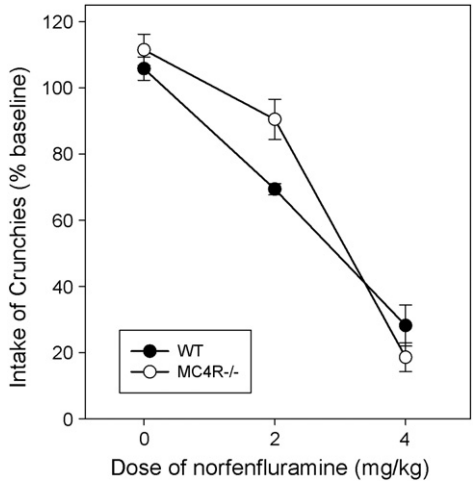

Fig. 1. Intake of Crunchies treat (expressed as mean ± SE of baseline) by groups of 5 wild type (WT) and MC4R−/− mice pretreated with various doses of dexnorfenfluramine. For WT, intakes at each dose all differ significantly. For MC4R−/−, intake after 4 mg/kg differs from vehicle (0 mg/kg). No pairwise comparison between groups was significant.

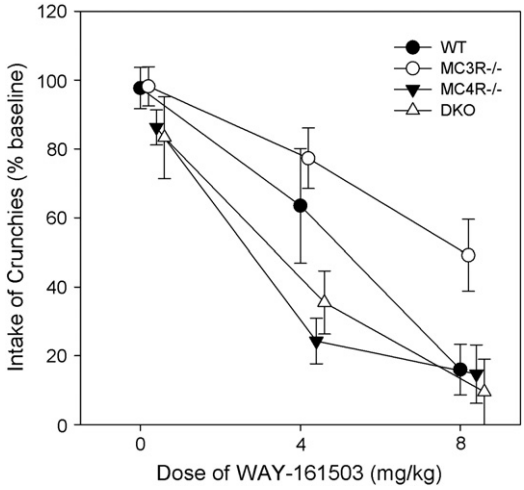

Fig. 2. Intake of Crunchies treat (expressed as mean ± SE of baseline) by groups of 6 WT, MC3R−/−, MC4R−/−, and DKO mice pretreated with various doses of WAY-161503. Intake of MC3R−/− differed significantly from all other groups at the highest dose. The four graphs are offset slightly at each dose to allow them to be more easily distinguished.

any of the groups, although MC3R−/− and DKO took the smallest meals.

The food intake results are shown in Fig. 2. Overall, WAY-161503 reduced intake of Crunchies in a dose-dependent manner. However, there were differences between groups at 4 mg/kg ($F_{3,20}=4.97$, $P=.01$) and 8 mg/kg (($F_{3,20}=4.03$, $P<.05$). Neither DKO nor MC4R−/− differed significantly from WT at either dose. Intake of MC3R−/− was higher than WT at the higher dose and from MC4R−/− and DKO at the lower dose ($Ps<0.05$). From Fig. 1, we estimate that the 50% inhibitory dose of WAY-161503 was ~2× higher in MC3R−/− than WT.

The results from the Fos measurements are shown in Fig. 3. One way ANOVAs, including the omnibus control group, showed significant group differences in PVN ($F_{4,20}=5.47$, $P=0.01$) and NTS ($F_{4,20}=2.81$, $P=.05$). Pairwise comparisons showed that in PVN, the DKO was greater than all other groups, and in NTS that MC4R−/− was greater than the omnibus control. Of note, WAY-161503 did not induce Fos above saline control in any region of either WT or MC3R−/−. Other brain regions, including the arcuate nucleus, were examined qualitatively and revealed no consistent group differences that merited closer inspection.

4. Discussion

In the first experiment, we found that DNOR produced full efficacy anorexia in MC4R−/− mice; although not supported statistically, there is a hint a possibility that they may be less sensitive at a lower dose. Highly effective doses of anorectic agents may have non-selective effects on eating behavior, including disruption of the behavioral satiety sequence; this was not measured in the present study but casual observation of the animals gave no indication of gross motor abnormalities. In addition to its presynaptic 5-HT-releasing effect, DNOR also has affinity for 5HT receptors, and notably the 2C subtype (Rowland and Carlton, 1986). Our data suggest that $5HT_{2C}$ receptor-mediated anorexia is independent of functional MC4R−/− signaling. The second experiment, using the $5HT_{2B/2C}$ agonist, WAY-161503 (Rosenzweig-Lipson et al., 2006), yielded consistent information in the MC4R−/− being at least as responsive to the anorectic effect as WT controls. In contrast, MC3R−/− mice had reduced sensitivity to WAY-161503. DKO mice showed the same behavioral response to WAY-161503 as MC4R−/−.

The results with MC4R−/− are at variance with interpretations in the literature and merit detailed discussion. Heisler et al. (2002) showed in rats that a threshold anorectic dose (1 mg/kg i.v.) of DFEN produced Fos labeling in a large fraction (45%) of α-MSH-expressing cells in the arcuate nucleus, and that ~80% of α-MSH cells expressed mRNA for $5HT_{2C}$ receptors. They also showed that DFEN doubled the firing rate of POMC cells in mouse hypothalamic slices. Their data show convincingly that DFEN will activate POMC neurons and

Table 1
Body weights and baseline intakes.

Group (*N*)	Body weight (g)	Baseline intake of Crunchies (g)
WT (+/+) (6)	28.0 ± 1.1^{a}	1.44 ± 11^{ab}
MC3R−/− (6)	25.2 ± 1.0^{a}	1.05 ± 04^{a}
MC4R−/− (6)	48.2 ± 2.8^{b}	1.58 ± 15^{b}
DKO (6)	38.7 ± 2.2^{c}	1.12 ± 06^{a}

Shown are Mean ± SE of the averages across 3 weeks for each mouse.
Intake of Crunchies has been transformed to g assuming 0.19 g/Crunchie.
Values with different superscript letters differ significantly ($P<0.05$, Tukey).

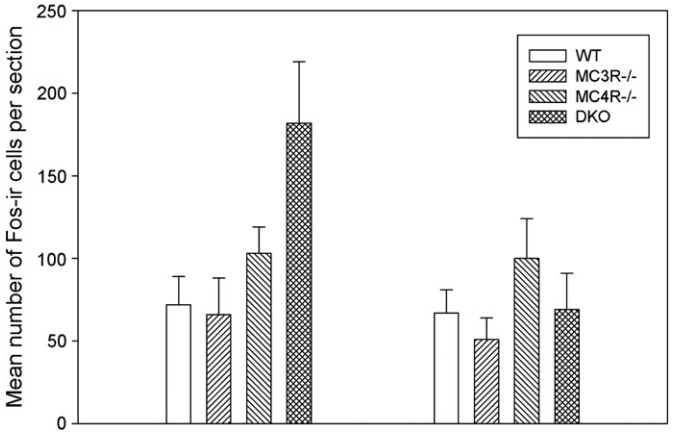

Fig. 3. Mean (+SE) numbers of Fos-ir cells in a typical section through paraventricular nucleus and nucleus of tractus solitarius in groups of 5 WT, MC3R−/−, MC4R−/− and DKO mice injected with WAY-161503 (8 mg/kg). The horizontal lines represent the mean values from an omnibus control group treated with vehicle. *$P<0.05$ differs from all other groups. The only groups that differed significantly from control were DKO (in PVN) and MC4R−/− (in NTS).

presumably release α-MSH from their terminals in PVN or other regions. To show a functional connection, Heisler et al. (2002) examined DFFEN anorexia in A^y mice that overexpress the endogenous melanocortin antagonist, agouti, and so are unresponsive to either endogenous or exogenous melanocortins. Non-deprived A^y mice were completely unresponsive to DFEN (3 mg/kg) in a test measuring intake of wet mash at the beginning of the dark cycle: WT mice showed ~50% suppression of intake in this test. In other studies, we have found that DNOR is ~2× more potent that DFEN (Rowland et al., 2000), so the dose used by Heisler et al. (2002) should be comparable to our lower dose. We found a slightly reduced effect in MC4R−/− relative to WT at this dose.

There are several possible explanations for the difference between our results and those of Heisler et al. (2002). First, DFEN and DNOR may not have identical anorectic effects. There is very little evidence for this and, if anything, DNOR should be more selective at $5HT_{2C}$ receptors (Rowland and Carlton, 1986). The second is that had we used a still lower dose of DNOR that was perhaps more selective for $5HT_{2C}$ receptors, we might have seen complete insensitivity, but that at higher doses additional mechanism(s) are engaged that are fully functional in MC4R−/− mice. However, as argued above, our lower dose (2 mg/kg) should have been in the same ballpark efficacy as 3 mg/kg DFEN. This is consistent with the observation that suppressions of intake in WT groups were comparable in Heisler et al. (2002) (~40%) and our study (a mean of 41% *vs* vehicle). The third possibility is that the critical receptors for the inferred release of α-MSH are type 3 rather than type 4. This distinction was not addressed by Heisler et al. (2002) who instead carefully referred to their result as indicating mediation through type 4 and type 3 receptors. In another study, Heisler et al. (2006) found that MC4R−/−mice did not suppress their nocturnal spontaneous food intake after dexfenfluramine (3 mg/kg), a dose that produced 50% inhibition in WT and MC3R−/−. Three possible differences include (i) spontaneous nocturnal intake may rely on different neural system(s) than elective daytime intake of a palatable food, (ii) that the juvenile and pre-obese mice used by Heisler et al. (2006) have significantly different brain organization than in the adult and obese condition, (iii) specificity of action of DNOR compared with other serotonergic agents. If the present and Heisler et al. (2006) results could be replicated in the same laboratory, this would both provide important insights on the limits of generality of these phenomena and their brain substrates.

Experiment 2 was designed to address the relative involvement of MC3 and MC4 receptors, and to use a more direct stimulation of $5HT_{2C}$ receptors. The results showed unequivocally that MC4R−/− were at least as responsive as WT to the anorectic action of WAY-161503. It could be argued that they received a higher absolute amount of drug since they weighed 172% of WT, but even adjusting the dose from mg/kg (Fig. 2) to absolute would not change the overall conclusion. In contrast, MC3R−/− showed reduced anorectic sensitivity to both doses of WAY-161503. Collectively, these data suggest that MC3R and not MC4R are the critical downstream targets of arcuate $5HT_{2C}$ activation. The data from DKO mice, which were fully responsive to WAY-161503, are not fully consistent with that interpretation. In particular, if MC3R are critical, then the DKO mice should have been as hyporesponsive as MC3R−/−. We have no current explanation for these data, unless WAY-161503 is acting at targets other than arcuate $5HT_{2C}$ receptors, at least in the knockout animals. This agent has strong selectivity to $5HT_{2C}$ (Ki ~ 6 nM) and to some extent $5HT_{2A}$ (Ki ~ 36 nM) receptors (Rosenzweig-Lipson et al., 2006).

The issue of receptor subtype was also addressed by Lam et al. (2008) who characterized the effect of a novel and selective 5-HT_{2C} receptor agonist BVT.X on food intake in MC4R null mice. Unlike our MC4R−/− mice which are a genomic knockout, Lam et al. (2008) used stock derived from a lox-TB MC4R null transgene animal developed by Elmquist and Lowell. They measured the spontaneous intake of powdered chow over the first 6 h of the night following acute injection of saline or BVT.X (K_i at 5-HT_{2C} receptors was reported as 9 nM with at least 100-fold selectivity over other 5-HT receptors). While BVT.X significantly reduced food intake (by ~50% in hours 2–5 after injection) in WT, it had no effect in MC4R null mice. There are differences between our protocol and that of Lam et al. (2008). First, they used mice soon after weaning at which time the 4R null mice had not become obese; it is possible that age and/or obesity modulate the effect of 5-HT agents. Obesity is unlikely to be a factor because in another experiment in their paper, Lam et al. (2008) showed that obese mice (either from high fat diet or ob/ob) showed similar anorexia (~50%) to lean WT at a dose of 60 mg/kg BVT.X. Age could be a factor: in other studies we have found that MC4R−/− mice at several months of age show normal anorectic responses to exogenous CCK (Vaughan et al., 2006) in contrast to the result of Fan et al. (2004) who found no anorectic response to CCK in MC4R−/− mice soon after weaning. If age turns out to be the critical factor, this is of both theoretical and translational importance. A second difference between our study and Lam et al. (2008) is the origin and method of production of the 4R null mice: it may be important to compare mice from both origins in the same laboratory. A third difference is the type of food and time of day of the anorexia tests. We doubt whether this is a major factor because we have compared the efficacy of DFEN in several feeding protocols and found small quantitative but not qualitative differences in drug efficacy (Rowland et al., 2000). A fourth possibility is that both DNOR and WAY-161503 are working through mechanisms that differ from BVT.X. The available data suggest that all of these agents should work through $5HT_{2C}$, but as with any pharmacological approach, agents may be acting at targets yet to be investigated. We note that the dose of BVT.X used (60 mg/kg) seems very high relative to equi-effective WAY-161503 (~4–8 mg/kg) given that their Kis at $5HT_{2C}$R are comparable.

We were surprised that WAY-161503 did not produce strong Fos activation in some brain regions, notably arcuate (not shown), PVN, and AP/NTS. These regions are strongly activated by DFEN and DNOR (Li and Rowland 1993; Rowland et al., 2000, 2003), and on the basis of both lesion and tolerance studies suggested that PVN activation may covary with serotonin-related anorexia (Li and Rowland 1996; Li et al., 1994; Rowland et al., 2003). Somerville et al. (2007), using a qualitative rating scale, reported that the 5-HT_{2C} agonist VER23779 induced Fos in relatively few brain regions of C57/Bl mice, although the effect in PVN was significant. In our WT mice, possibly a higher dose or larger N may have produced a significant effect (Fig. 3). However, DKO did show activation in PVN under these conditions: this could be interpreted as an absence of inhibition (mediated by both MC3R and MC4R) but leaves unclear the nature of the stimulation. We know from the one DKO animal in the omnibus control group as well as in other unpublished studies that DKO do not have abnormally high constitutive Fos in PVN (or other areas) and not an abnormally high response to handling and saline injection.

MC3R are found in many regions of the forebrain (Lindblom et al., 1998) and among other sites appear to act as inhibitory autoreceptors on POMC neurons (Lee et al., 2008). Thus, the MC3R−/− may have several adaptations within MC circuits as well as outside such circuits. This should also be considered in light of the present findings. Thus, 5-HT_{2C} agonists may be less active in MC3R−/− because the basal firing rate or other aspects of excitability of POMC neurons has changed. Similar difficulties of interpretation have been met by others, including Nonogaki et al.(2009) on the action of a 5-HT uptake blocker on POMC neurons in $5HT_{2C}$ knockout mice. It should also be borne in mind that the forebrain and notably arcuate nucleus–PVN axis is most likely not the only important site of MC action for anorexia. Thus, MC4Rs are present on afferent vagal fibers in the NTS (Wan et al., 2008), and 5-HT agonists induce Fos-ir in NTS neurons that are predominantly catecholaminergic (Lam et al., 2009). In this regard, while the stimulation of Fos-ir in NTS by WAY-161503 was not particularly strong (Fig. 2), it was significantly above control levels in only the MC4R−/− group.

In summary, our data suggest that MC4R are not a critical downstream target for the anorectic action of serotonergic agents in adult mice. Instead, MC3R may be a target. In this regard, one likely downstream, target region, the PVN, expresses both type 3 and 4 receptors, so this is anatomically feasible. Possible reasons for the discrepancy between the studies were discussed but no single factor can be identified at this time, and further studies are warranted.

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