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Functional mutations in mouse norepinephrine transporter reduce sensitivity to cocaine inhibition

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Summary

The transporters of dopamine, norepinephrine and serotonin are molecular targets of cocaine, amphetamine, and therapeutic antidepressants. The residues involved in binding these drugs are unknown. We have performed several rounds of random and site-directed mutagenesis in the mouse norepinephrine transporter and screened for mutants with altered sensitivity to cocaine inhibition of substrate uptake. We have identified a triple mutation that retains close to wild-type transport function but displays a 37-fold decrease in cocaine sensitivity and 24-fold decrease in desipramine sensitivity. In contrast, the mutant's sensitivities to amphetamine, methamphetamine, and methylphenidate are only slightly changed. Our data reveal critical residues contributing to the potent uptake inhibitions by these important drugs. Furthermore, this drug-resistant triple mutant can be used to generate a unique knock-in mouse line to study the role of norepinephrine transporter in the addictive effects of cocaine and the therapeutic effects of desipramine.

Keywords

norepinephrine transporter; dopamine transporter; cocaine; antidepressants; random mutagenesis

Introduction

Cocaine addiction is a devastating social and health problem. Cocaine produces its powerful effects primarily by binding to and inhibiting the functions of three important proteins, the dopamine (DA) transporters (DAT), norepinephrine (NE) transporters (NET), and serotonin transporters (SERT) (Amara and Kuhar, 1993). These transporters clear neurotransmitters from the synapses and surrounding areas by the reuptake and recycling of the monoamines. Therefore, they play crucial roles in regulating the monoamine neurotransmissions which involve many important brain functions (Rothman and Baumann, 2003). Cocaine inhibition of these transporters results in prolonged elevation of the three monoamines in various brain regions. It is not very clear how each of these transporters contribute to the complex effects of cocaine. Genetically modified mice with each of these transporter genes singularly deleted

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exhibit the rewarding effects of cocaine, suggesting that no single transporter is required for cocaine reward in these knockout mice (Rocha et al., 1998; Sora et al., 1998; Xu et al., 2000). However, the knockout mice have profound adaptive changes in response to the absence of important gene products during development, which makes it difficult to interpret data from these mice. Therefore, we generated a knock-in mouse line with a functional, but cocaine resistant, DAT mutant (Chen et al., 2006). Cocaine no longer produces reward in these mice, demonstrating that cocaine inhibition of a functional DAT is required for its rewarding property in mice. However, it is not known whether DAT inhibition is sufficient for cocaine reward or how cocaine actions on NET and SERT contribute to cocaine effects. In this study, we sought to identify residues in NET that are critical for potent cocaine inhibition and to engineer a NET mutant that retains transport function but with substantially reduced cocaine sensitivity.

In 2005, the crystal structure of a leucine transporter from an *Aquifex aeolicus* bacterium (LeuTaa) was solved (Yamashita et al., 2005). LeuTaa belongs to the Na⁺- and Cl⁻-dependent neurotransmitter transporter family including NET, DAT, and SERT. However, the solved crystal structure likely represents one of many conformations of a transport cycle. Furthermore, LeuTaa and the neurotransmitter transporters only share 25% conserved residues and, most importantly, cocaine does not bind to LeuTaa. It remains unclear how cocaine inhibits the monoamine transporters and which residues are involved.

Previously, we reported the identification of residues important for cocaine inhibition of DAT and SERT by comparing transporters from mouse and from insects (Gu et al., 2006; Wu and Gu, 2003). We then generated a mutant of mDAT with triple mutations in the transmembrane domain 2 (TM2) that is functional but 69-fold less sensitive to cocaine inhibition than wild-type (WT) mDAT (Chen et al., 2005). In this study, we made similar mutations in TM2 of mNET and performed several rounds of random and site-specific mutagenesis in TM2 and TM3. We identified several residues important for cocaine inhibition and engineered a mutant mNET that retains near WT transport function but is 37-fold less sensitive to cocaine inhibition and 24-fold less sensitive to desipramine inhibition.

Methods

Materials

The WT mNET cDNA was described previously (Han and Gu, 2006). [³H]-DA (28 Ci/mmol) was purchased from PerkinElmer (Boston, MA). [³H]-NE (38 Ci/mmol) was purchased from GE Healthcare (Piscataway, NJ). Dopamine, norepinephrine, nisoxetine, and desipramine were from Sigma-Aldridge (St. Louis, MO). Oligonucleotide primers were synthesized by commercial DNA synthesis services (Integrated DNA Technologies, Coralville, IA and Sigma-Genosys, St. Louis, MO).

Site-directed random mutagenesis

Mutagenic primers utilizing codon SNN (S: G or C; N: A, T, C, or G) were used in PCR reactions to introduce random mutations to all 20 amino acids at the specific residues of mNET. Standard techniques of molecular biology were used. Selected mutant clones were sequenced to determine the exact mutations.

Functional analysis

Transient transfection and uptake assays were performed as described (Gu et al., 2006; Wu and Gu, 2003). Briefly, the mNET and mutants cDNA were subcloned into the bluescript vector SKii⁺ (Stratagene, La Jolla, CA) which was under the control of a T7 promoter. HeLa cells (American Type Culture Collection, Rockville, MD) were infected with recombinant vTF-7 vaccinia virus which carries the T7 polymerase gene, and transiently transfected with the

plasmid bearing mNET or mutants cDNA using Lipofectin (Life Technologies, Gaithersburg, MD). To determine IC_{50} values, cells were incubated in Phosphate Buffered Saline with 1mM $CaCl_2$ and 0.1mM $MgCl_2$ (pH 8.0) (PBS/Ca/Mg) containing 40nM [3H]NE or [3H]DA in the presence of different concentrations of inhibitor. To determine K_m and V_{max} values, cells were incubated in PBS/Ca/Mg buffer containing 100 nM [3H]NE or [3H]DA in the presence of increasing concentrations of unlabeled (0.1–10 μ M) NE or DA for 10 min at 22°C. Reactions were terminated by two successive washes with PBS/Ca/Mg. Amounts of [3H]NE or [3H]DA accumulated in the cells were quantitated by liquid-scintillation counting (Topcount; Packard Instrument Company, Meriden, CT). All experiments were performed in triplicate. Mock-transfected cells were used as controls and radioactivity associated with these cells was considered the background and subtracted from the total uptake. The amounts of [3H]NE and [3H]DA accumulated in the cells were quantitated by liquid-scintillation counting (Topcount, Packard Instrument Company, Meriden, CT). All experiments were performed in triplicate. K_m , V_{max} , and IC_{50} values were calculated by non-linear regression analysis of the data using Prism 3 (GraphPad Software, San Diego, CA).

Cell Surface Biotinylation

Two hemagglutinin (HA) tags (YPYDVDPDYAYPYDVDPDYA) were fused to the 5' end of WT and each mutants NET. Fusion proteins were tested for uptake activity as described above and for expression by immunoblotting with a HA tag antibody (H9658, Sigma-Aldridge). The procedure for the cell surface protein detection was similar to that previously described (Gu et al., 1996). Briefly, cells were plated in six-well plates at a full confluence and transfected with WT or a mutant mNET cDNA construct as described above. After washing with 2 mL of cold PBS/Ca/Mg, cells were treated with 1.5 mg/mL sulfo-NHS-SS-biotin (Sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate, Pierce, Rockford, IL) in PBS/Ca/Mg (pH 8.0) for 20 min at 4°C. The biotinylation process was further repeated with a fresh addition of sulfo-NHS-SS-biotin in PBS/Ca/Mg at pH 8.5. Free sulfo-NHS-biotin was removed by washing twice with 4°C 0.1 mM glycine in 1 mL PBS/Ca/Mg. The reaction was further quenched by incubation with 0.1 mM glycine for 30 min at 4°C and cells were then washed twice with PBS/Ca/Mg. Cells were dissolved in the lysate buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100, 1.0% sodium deoxycholate) containing Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Cell debris was removed by sedimentation at 15,000×g for 30 min. A fraction of the cleared whole cell lysate was saved for the analysis of the total amount of NET. The biotinylated proteins from the remaining lysate (750 μ L) were recovered by adding 150 μ L of 50% slurry of avidin-agarose beads (Pierce Biotechnology, Rockford, IL) to the cell lysates and were incubated overnight with gentle agitation at 4°C. After three washes with the lysis buffer, the proteins bound to the beads were eluted in 100 μ L Laemmli sample buffer containing 100 mM dithiothreitol (DTT). The total cell lysates and eluted surface proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then probed with mouse monoclonal anti-HA antibody (H9658, Sigma-Aldridge) with a 1:2000 dilution in a PBS/0.05% Tween-20/10% low fat milk blocking buffer. The blot was washed with blocking buffer then incubated with secondary anti-mouse IgG, peroxidase-conjugated whole antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with a 1:2000 dilution in blocking buffer and then visualized by Enhanced Chemiluminescence (32106, Pierce Biotechnology). The densities of the protein bands were determined using ImageJ 1.38X (NIH, Bethesda, MD).

Statistical Analysis

Student's t-test values were performed to assess the significance of the difference between the WT mNET and the mutants.

Results

Specific mutations

We have previously reported that single mutation F105C and triple mutation L104V-F105C-A109V in mDAT increase cocaine IC_{50} by 15-fold and 69-fold (Chen et al., 2005; Wu and Gu, 2003). Since mDAT and mNET are highly homologous, residues involved in cocaine inhibition on the two transporters are likely to be similar. Therefore, we first made these corresponding mutants in mNET, F101C and L100V-F101C-A105V. However, these two mutants only exhibited modest decrease in cocaine sensitivity, with 4.5- and 8.4-fold increase in IC_{50} values (Table 1).

First round of random mutagenesis

Because these specific mutations in mNET did not alter cocaine sensitivity sufficiently, we employed random mutagenesis to screen for mutants with larger changes in cocaine inhibition. In addition to F105C in TM2, it was reported that mutation F154A in DAT TM3 decreases cocaine sensitivity by 10-fold compared to the WT DAT (Lin and Uhl, 2002). It is likely that the corresponding residues in mNET, F101 and F150, would also contribute to cocaine inhibition. Therefore, our first round of random mutagenesis was aimed at point mutations at these two residues and residues in close proximity (residues 97–106 in TM2 and residues 146–154 in TM3) based on the alpha-helix model and sequence alignment with LeuTaa (Figure 1).

Primers encoding all 20 amino acids at a particular residue were used in PCR reactions to generate a small library of random mutants at each targeted position in mNET. Individual mutant clones were transiently transfected into cultured cells grown in 96-well plates and screened for functional transport and for decreased sensitivity to cocaine inhibition using WT mNET as control for both measurements. Uptake activities in the presence and absence of 1 μ M cocaine were determined and the ratios of the two were used as estimates of the cocaine sensitivities for the mutant clones.

The results are shown in Table 2 along with the residues mutated, the percent of the screened clones that retained greater than 50% uptake activity, and the number of clones that displayed decreased or increased cocaine sensitivity. Approximately 96 clones were screened for most mutant residue constructs. The results in Table 2 suggest that significant portions of random mutations at P97 in TM2, Y151 and N153 in TM3 were tolerated and some of them displayed decreased sensitivity to cocaine inhibition. Clones with desired properties were sequenced to determine the exact mutations. We found that mutating P97 to Alanine or Serine increased the cocaine IC_{50} values by 11- or 7-fold, respectively, and mutating N153 to Threonine increased the IC_{50} by 10-fold compared to WT mNET (Table 1). These mutants also retained significant uptake activity. Therefore, we focused on the two single mutants N153T and P97A and used them as the base for further mutagenesis. Interestingly, mutation A105C did not decrease cocaine sensitivity but appeared to increase uptake activity (Table 1) by decreasing the K_m value (data not shown).

Second round of random mutagenesis

The second round of mutagenesis was to introduce random mutations at a residue in close proximity to N153T or P97A. Double mutations with N153T and a random mutation at G149, Y152, S154, I156, and A157 were relatively well tolerated, but none of the double mutations further decreased cocaine sensitivity. Double mutations in TM2 containing P97A were not tolerated (data not shown). Next we generated double mutants combining one mutation in TM2 and another in TM3 of mNET. A large number of such double mutants were screened. We identified a number of clones that displayed significant further reductions in cocaine sensitivity. When N153T was combined with mutations in TM2, A105L, F101S, or F101M, transporter

activity was comparable to the N153T single mutant, but cocaine IC_{50} values increased by 30, 40, 25-fold respectively. The double mutant F101C-N153T had a cocaine IC_{50} value that was 76-fold higher than that of WT mNET, but the substrate uptake activity further decreased to 35% of the WT mNET (Table 1).

Third round of random mutagenesis

The observation that mutation A105C had enhanced uptake suggested the possibility that mutations at certain residues may improve the functional property of F101C-N153T while retaining cocaine insensitivity. Therefore, we made triple mutations having F101C-N153T and a random mutation at a third residue in TM2 or TM3. Most of the triple mutants with the third mutation in TM3 did not improve transport function. In contrast, more than 50% of triple mutants F101C-N153T-L100X (the third mutation in TM2) improved transport function to greater than 50% of the WT mNET. Surprisingly, these mutants did not retain the cocaine insensitivity of F101C-N153T. Finally, we identified a triple mutant F101C-A105G-N153T that retained 76% uptake activity but was much less sensitive to cocaine with an IC_{50} value 37-fold higher than that for WT mNET (Table 1).

Characterization of the triple mutant F101C-A105G-N153T

The triple mutant F101C-A105G-N153T (mNET_{CGT}) was further analyzed for its sensitivities to other drugs. Figure 2 shows the results of representative experiments comparing mNET and mNET_{CGT} in uptake inhibition by the psychostimulants cocaine and methylphenidate (Ritalin), the NET substrates amphetamine and methamphetamine, and the NET selective inhibitors desipramine and nisoxetine. The IC_{50} values indicated that the triple mutation did not change substantially the transporter's sensitivity to methylphenidate, amphetamine, and methamphetamine inhibition but increased the IC_{50} values for cocaine, desipramine, and nisoxetine by 37-, 24-, and 8-fold, respectively.

The transport kinetics for mNET and mNET_{CGT} were determined with NE and DA as substrates because in certain brain regions, and/or under certain circumstances, DA could be cleared by NET. Figure 3 shows the results of representative experiments. The K_m values of NE uptake were not significantly different ($p > 0.05$, t-test). While the V_{max} value for mNET_{CGT} appeared to be lower than mNET, the difference did not reach the criterion of statistical significance ($p > 0.05$, t-test). When DA was used as substrate, the K_m value for mNET_{CGT} (408 nM) was significantly larger than that for mNET (277 nM, $p < 0.05$, t-test). However, the differences between the V_{max} values were not significant.

To determine whether the triple mutation altered transporter expression pattern, we measured cell surface expression of mNET_{CGT} and mNET using the membrane-impermeable biotinylation reagents. Figure 4 shows a representative western blot for mNET and mNET_{CGT}. Similar banding patterns were observed between the wild-type mNET and mNET_{CGT}, with a well defined band at 65 kDa and broad bands around 120 kDa, consistent with results from other investigators (Dipace et al., 2007). There were no significant differences between the two constructs for either the total transporter expression or the biotinylated surface transporter expression ($p > 0.05$, t-test).

Discussion

Cocaine binds and inhibits all three monoamine transporters, DAT, NET, and SERT, from human and mouse with similar potencies (Han and Gu, 2006). An abundance of evidence is consistent with the notion that cocaine inhibition of DAT and subsequent elevation of extracellular DA plays the major role in mediating cocaine effects (Kuhar et al., 1991; Wise and Bozarth, 1987). However, DAT knockout mice still self-administer cocaine and display

cocaine conditioned place preferences (Rocha et al., 1998; Sora et al., 1998). These studies show that DAT is not required for the rewarding effect of cocaine in certain circumstances. Since adaptations in DAT knockout mice may alter the reward circuitry, our lab generated a knock-in mouse line with a functional, but cocaine resistant, DAT mutant. Cocaine no longer produces reward in these mice, suggesting that DAT inhibition is required in mice with a functional DAT (Chen et al., 2006). However, it is not known whether DAT inhibition is sufficient for cocaine reward and it is not clear how NET and SERT contribute to the complex cocaine effects. Some studies have shown that the NE system and NET also play critical roles in producing cocaine effects (Ventura et al., 2007). Since NET knockout mice may also have adaptive changes resulting in different cocaine responses from those in normal mice, we plan to generate a knock-in mouse line with a cocaine resistant NET to study the contribution of NET to cocaine effects. Despite intensive studies, the residues critical for potent cocaine inhibition remains unknown.

Previously, we reported that single mutation F105C and triple mutation L104V-F105C-A109V in mDAT TM2 increase cocaine IC₅₀ by 15-fold and 69-fold respectively. In this study, we made the corresponding mutations in mNET which displayed 4.5-fold and 8.4-fold increase in cocaine IC₅₀ values (Table 1) respectively. This result suggests that cocaine binding sites in DAT and NET are similar, but not exactly the same. It is possible that different mutations at these residues may result in mutants with increased cocaine resistance. We screened for such random mutants and were unsuccessful.

As shown in Table 2, a fraction of random mutants at all selected residues except Y152 retained 50% or higher transport function, suggesting that changes at these positions can be tolerated but Y152 is critical for transport function. It has been shown that Y108 in LeuTaa, the corresponding residue of mNET Y152 in mNET, is at the presumed substrate binding site, which explains why Y152 is critical. Table 2 also shows that random mutations at residues I104, A105, L106, Y107 and G149 had high percentage of clones with high uptake activity, indicating that these positions do not require specific residues to maintain transport functions. In contrast, very few random mutants showed altered cocaine sensitivity except those at P97 and N153, suggesting that changes at most of the residues have little effect on cocaine inhibition and P97 and N153 may be involved in cocaine binding directly or indirectly.

It is worth noting that random mutations P98X produced five mutants with cocaine sensitivity increased nearly 3-fold. In addition, more than 50% of triple mutants F101C-N153T-L100X (the third mutation in TM2) improved transport function to over 50% of the WT mNET. Surprisingly, these mutants did not retain the cocaine insensitivity of F101C-N153T. In other words, the additional mutations at L100 increased cocaine sensitivity of L101C-N153T and enhanced transport function. Further studies with computer models are needed to reveal how L100 affects both cocaine inhibition and substrate uptake.

Double mutations within TM2 or TM3 did not result in mutants with further decrease in cocaine sensitivity; but, combined TM2 and TM3 double mutations did, suggesting that changes on both TM2 and TM3 have additive effects. Another interesting observation is that some mutations at A105 exhibited a decreased K_m when compared to WT mNET (data not shown) and thus enhanced transport function. The single mutant A105C appeared to increase substrate uptake activity while A105G in the triple mutation mNET_{CGT} rescued the partial loss of transport function by the mutations of F101C and N153T.

The triple mutant L104V-F105C-A109V in mDAT was generated for cocaine resistance but it is also insensitive to methylphenidate, which suggests that these mutations affect uptake inhibition by the two psychostimulants in a similar fashion. However, triple mutation F101C-A105G-N153T in mNET reduced sensitivity to cocaine but only slightly to methylphenidate.

As shown in Figure 2, this triple mutation did not change the IC₅₀ values for amphetamine and methamphetamine. This is expected because the two drugs are analogs of mNET substrates, NE and DA, and because we screened and selected mutants that retained transport function.

In summary, we have performed several rounds of random mutagenesis in mNET and screened for mutants that have decreased cocaine sensitivity while retaining transport function. We identified a triple mutant, F101C-A105G-N153T, containing two mutations in TM2 and one mutation in TM3. This mutant retained close to WT transport function but displayed a 37-fold decrease in cocaine sensitivity and 24-fold decrease in desipramine sensitivity. In contrast, the mutant's sensitivities to amphetamine, methamphetamine, and methylphenidate are only slightly changed. Our data reveal a number of residues that are important for potent cocaine inhibition. Furthermore, this triple mutant can be used to generate a unique knock-in mouse line to study the role of NET in the addictive effects of cocaine and the therapeutic effect of desipramine.

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Fig. 1. Two dimensional serpentine schematic of the mNET TM2 and TM3

The TM segments are based on sequence alignment with the LeuTaa crystal structure and its alignment with the Na^+/Cl^- coupled transport proteins. The F101C-A105G-N153T positions of the cocaine-insensitive mNET mutant are highlighted with a larger font and bold circles.

Fig. 2. Drug inhibitions of mNET_{CGT} and wild-type mNET

HeLa cells were transiently transfected with the wild-type and mutant mNET cDNAs and incubated in a buffer containing 40 nM [³H]NE and each of 6 different drugs at concentrations indicated. The uptake activities are presented as fractional activities relative to those in the absence of drugs. Each data point is expressed as mean $\pm$ SEM (n=3). The experimental data were fitted using non-linear regression analysis. The 6 drugs tested were: A) Cocaine; B) Methylphenidate (Ritalin); C) Amphetamine; D) Methamphetamine; E) Desipramine; and F) Nisoxetine.

Fig. 3. Transport kinetics of the wild-type and mutant mNET

HeLa cells were transfected with the mNET and mNET_{CGT} cDNAs. Transport activities were measured in assay buffers containing 100 nM [³H]NE or [³H] DA and increasing concentrations of unlabeled NE (A) or DA (B). Transport rate (pmol/min/mg protein) was plotted vs. total NE or DA concentration. Each data point is expressed as mean ± SEM (n=3).

Fig. 4. Cell surface expression levels of mNET and mNET_{CGT}

Intact cells transiently transfected with the mNET and mNET_{CGT} cDNAs were incubated with membrane impermeable a biotinylation reagent. The cells were then lysed and the biotinylated proteins were purified with avidin-agarose beads. The proteins were analyzed with Western blot. The first two lanes are whole cell lysates from cells transfected with mNET_{CGT} and mNET cDNAs, respectively, with the same amount of proteins loaded. The last two lanes are biotinylated proteins purified from the same amount of cell lysates transfected with mNET_{CGT} and mNET. Molecular mass markers are indicated at the right of the blot.

Table 1

Cocaine sensitivity and uptake activity of the wild-type and mutant mNET.

Constructs	^a Cocaine IC ₅₀ , μ M	^b Uptake (% WT)
mNET	0.35 $\pm$ 0.02	100%
F101C	1.4 $\pm$ 0.05	73%
L100V-F101C-A105V	2.6 $\pm$ 0.4	65%
P97A	3.4 $\pm$ 0.3	65%
N153T	3.2 $\pm$ 0.1	49%
A105C	0.41 $\pm$ 0.10	110%
F101C-N153T	23.7 $\pm$ 3.6	35%
F101C-A105G-N153T	12.9 $\pm$ 0.9	76%

^a HeLa cells were transiently transfected with the wild-type and mutant mNET constructs. NE uptake activities were measured in the presence of different concentrations of cocaine as described in Figure 2 and the IC₅₀ values of cocaine inhibition of NE uptake were determined with the software Prism 3.

^b Uptake activities of the mNET mutants were measured in assay buffer containing 200nM NE ([³H] labeled and unlabeled) and are presented as relative to that of WT mNET (100%).

Table 2

Summary of the first round of random mutagenesis in TM2 and TM3 of mNET.

mNET mutants	% active ^b	decreased cocaine sensitivity ^c	Increased cocaine sensitivity ^d
P97X ^a	52%	17	0
Y98X	13%	0	5
L100X	19%	0	0
F101X	14%	0	0
I104X	53%	0	0
A105X	48%	0	0
L146X	75%	0	0
Y147X	67%	0	0
V148X	16%	0	0
G149X	52%	1	1
F150X	32%	1	0
Y151X	37%	9	0
Y152X	0%	0	0
N153X	34%	35	0
S154X	25%	0	1

^aP97X denotes mNET mutants at residue 97 were changed from P to any of the 20 amino acids. Other mutants are named in the same manner.

^bPercent of the screened clones that retained 50% or higher uptake activity.

^c or ^d Number of mutant clones with an estimated cocaine IC₅₀ value that was 3 fold as high or 1/3 of the IC₅₀ value for the WT mNET.