New inducible genetic method reveals critical roles of GABA in the control of feeding and metabolism

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Currently available inducible *Cre/loxP* systems, despite their considerable utility in gene manipulation, have pitfalls in certain scenarios, such as unsatisfactory recombination rates and deleterious effects on physiology and behavior. To overcome these limitations, we designed a new, inducible gene-targeting system by introducing an in-frame nonsense mutation into the coding sequence of Cre recombinase (nsCre). Mutant mRNAs transcribed from *nsCre* transgene can be efficiently translated into full-length, functional Cre recombinase in the presence of nonsense suppressors such as aminoglycosides. In a proof-of-concept model, GABA signaling from hypothalamic neurons expressing agouti-related peptide (AgRP) was genetically inactivated within 4 d after treatment with a synthetic aminoglycoside. Disruption of GABA synthesis in AgRP neurons in young adult mice led to a dramatic loss of body weight due to reduced food intake and elevated energy expenditure; they also manifested glucose intolerance. In contrast, older mice with genetic inactivation of GABA signaling by AgRP neurons had only transient reduction of feeding and body weight; their energy expenditure and glucose tolerance were unaffected. These results indicate that GABAergic signaling from AgRP neurons plays a key role in the control of feeding and metabolism through an age-dependent mechanism. This new genetic technique will augment current tools used to elucidate mechanisms underlying many physiological and neurological processes.

inducible gene knockout | feeding behavior | eating disorders | GABA | AgRP neurons

In experimental systems, there are many instances in which one would like to inactivate (or activate) a gene in adult animals to ascertain its function and avoid early developmental influences. A common strategy involves making conditional alleles of the gene of interest by flanking critical exons with loxP sites and then breeding those mice with mice expressing an inducible Cre recombinase. The tamoxifen-based Cre^{ER} and RU486-based Cre^{PR} systems are two popular tools that are used to achieve inducible control of gene expression (1, 2). However, these systems possess some caveats in addressing fundamental biological issues, such as silencing effects, as well as Cre- and inducer-mediated cytotoxicity (3–6). We and others have found that, in many cases, the inducibility of Cre^{ER} and Cre^{PR} systems was partially or even completely silenced, suggesting that estrogen receptor (ER)-binding or progesterone receptor (PR)-binding domains may negatively affect the expression of these Cre fusion proteins (5–8). We generated an agouti-related peptide *Agrp–*Cre^{ER} line of mice by gene targeting, but the gene was completely silenced and could not be activated with tamoxifen or the active metabolite 4-OH-tamoxifen, whereas when *Cre* alone was targeted to the same location within the *Agrp* gene, it functioned as expected (9, 10). However, BAC transgenic mice with a functional *Agrp–*Cre^{ER} have been described suggesting that chromosomal location influences silencing (11).

In-frame, nonsense mutations that generate premature termination codons (PTCs) in mRNA coding regions account for a sizable portion of human genetic diseases, such as cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD) (12). In the absence of any intervention, the majority of PTC-containing mRNAs is rapidly degraded by the process of nonsense-mediated decay (NMD), whereas only a small fraction of the transcripts can be translated into truncated, nonfunctional peptides (13). Aminoglycosides, such as gentamycin and geneticin (G418), protect mutant transcripts from NMD and promote read-through of PTCs (14–16). This process, termed nonsense suppression, has been shown to restore synthesis of full-length, functional proteins in numerous cell culture assays, animal models of human diseases, and even in patients with DMD or CF (17–19).

To better understand the genetic mechanisms underlying complex brain functions, we designed a novel inducible gene-targeting system by introducing an in-frame nonsense mutation into the coding sequence of the Cre recombinase gene. Mutant mRNAs transcribed from *nsCre* transgene can be efficiently translated into full-length, functional Cre recombinase in the presence of nonsense suppressors. In this work, we applied this new technique to acutely inactivate GABA signaling by neurons in the hypothalamus that are important regulators of feeding and metabolism.

Results

We conceived a new, inducible Cre-expression system by engineering an in-frame nonsense mutation (TGA) into the coding region of the *Cre* recombinase gene (*nsCre*) with the expectation that treatment with an aminoglycoside would promote read-through

Significance

We established a new genetic method for rapidly activating Cre recombinase that is based on suppression of a nonsense codon within Cre by a new generation of aminoglycosides. We applied this strategy to successfully silence both enzymes responsible for GABA biosynthesis in hypothalamic agouti-related peptide (AgRP) neurons that control appetite and other behaviors. Inactivation of GABA signaling in AgRP neurons of young adult mice resulted in severe loss of body weight and abnormal glucose metabolism, whereas older mice only manifested transient appetite deficiency. This genetic system adds another robust tool that can be used to understand complex neurological processes.

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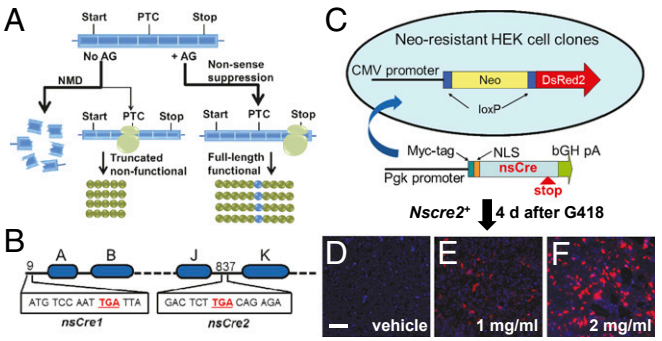

Fig. 1. Designing and validation of a novel inducible *nsCre* transgene. (*A*) A schematic diagram showing the aminoglycoside (AG)-mediated nonsense suppression of the PTC. Without treatment of AG, the majority of PTC-bearing mRNAs are rapidly degraded by the process of NMD, whereas only a small fraction of the transcripts can be translated into truncated, nonfunctional peptides. In contrast, AG, such as geneticin (G418), promotes read-through of PTCs during the translation, thereby restoring full-length functional proteins. Of note, a random amino acid (blue spheres) is incorporated into the PTC locus through AG-mediated nonsense suppression. (*B*) A PTC, TGA, was inserted into position 9 or 837 of the wild-type *Cre* gene. (*C*) A diagram illustrating the functional validation of *nsCre* transgene in a cell culture-based assay. Neo-resistant HEK293 cells were transfected with *CMV-flox-Neo-dsRed2* and *Pgk-myc-nls-nsCre* plasmids. (*D–F*) Representative images showing DsRed fluorescence in nsCre2-expressing HEK293 cells 4 d after treatment of vehicle (*D*) or G418 at a concentration of either 1 mg/mL (*E*) or 2 mg/mL (*F*). These results indicated that *nsCre*-mediated homologous recombination is restored by G418 in a dose-dependent manner. (Scale bar, 200 μm.)

of this nonsense codon, thereby allowing synthesis of functional Cre recombinase that could mediate recombination of *loxP*-flanked genes to inactivate their expression. Aminoglycosides lead to mispairing of a near-cognate aminoacyl-tRNA with a PTC, so that one of a specific set of amino acids can be incorporated, thus preventing termination (Fig. 1*A*) (16). Hence, to produce a functional Cre recombinase, the protein must tolerate the random incorporation of an exogenous amino acid at the PTC site. Evidence from a large-scale, random transposon insertion/deletion analysis revealed that regions preceding α-helix A and between α-helices J and K in the *Cre* coding sequence confer significant tolerance to insertional mutagenesis (20). Based upon these data, we designed a cell-culture recombination assay to validate candidate *nsCre* transgenes in which a TGA stop codon was inserted into either one of these two regions (Fig. 1*B*). We chose the TGA codon over the other two stop codons because evidence from previous studies indicated that UGA in mRNA exhibits the highest rate of read-through (15–17, 21). Neo-resistant HEK293 cells were cotransfected with *CMV-loxP-Neo-loxP-DsRed2*, along with *Pgk-nsCre* plasmids with stop codons inserted into one of the two positions. The next day, the cells were treated with increasing doses of the aminoglycoside G418 (Fig. 1*C*). Four days after drug treatment, DsRed2 fluorescence indicated that nonsense suppression had occurred in a dose-dependent manner when PTC was inserted into the region between α-helices J and K (*nsCre2*), but not ahead of α-helix A (*nsCre1*) (Fig. 1 *D–F*). In the case of *nsCre1*, functional Cre recombinase was produced without G418 treatment, presumably because translation started at a downstream, in-frame initiation codon. These results were confirmed with an assay in which an HEK293 clone with stable expression of *CMV-loxP-Neo-loxP-DsRed2* was transfected with the *Pgk-nsCre2* plasmid and treated with various doses of G418. Short exposure to G418 (2 mg/mL) followed by a recovery period without the aminoglycoside resulted in almost as many fluorescent cells as the transfection with the wild-type *Pgk-Cre* plasmid.

To establish a proof-of-concept mouse model, an *nsCre2*-containing transgene was targeted to the *Agrp* gene locus to generate $Agrp^{nsCre}$ mice, and these mice were subsequently bred with a Cre-dependent fluorescence reporter line ($Rosa26^{tdTomato}$) (22), in which removal of a loxP-flanked Neo gene allows expression of tdTomato (Fig. 2*A*). After injection of saline into the third ventricle of $Agrp^{nsCre/+}::Rosa26^{tdTomato}$ mice, tdTomato fluorescence was undetectable throughout the whole brain, indicating that *nsCre2* was completely silent and that no recombination of the reporter gene had occurred (Fig. 2*B*). In contrast, administration of G418 into the third ventricle led to robust, dose-dependent activation of tdTomato in the arcuate nucleus (ARC), where AgRP neurons are located (Fig. S1 *A* and *E*). These results suggested that G418 allows translational read-through of PTC in *nsCre2* transcripts, resulting in functional Cre recombinase activity in vivo. However, the toxicity of G418 to eukaryotic cells limits its application to proof-of-concept experiments, as shown in several genetic disease models (17, 21). Based upon an idea that the structural elements in aminoglycosides that induce PTC read-through are separable from those that affect toxicity (20), we designed and synthesized a series of G418 derivatives, including NB124, NB127, and NB128 (Fig. S2). These lead compounds displayed significantly reduced cytotoxicity while retaining equal or better read-through capacity compared with G418 (23). Based upon toxicological assays, NB124, NB127, and NB128 displayed ∼30% of the cytotoxicity compared with G148, while maintaining comparable potency in cell-culture assays. We tested the read-through capacity in vivo by injection of each of the compounds into the third ventricle of $Agrp^{nsCre/+}::Rosa26^{tdTomato}$ mice. Among these compounds, NB124 displayed superior read-through capacity in vivo as indicated by the number of tdTomato-expressing neurons in the ARC 4 d after drug treatment (Fig. S1 *B–D* and *F–H*). More importantly, a series of dose–response experiments resulted in a regimen in which treatment with NB124 resulted in tdTomato fluorescence in ∼95% of AgRP neurons, suggesting that $Agrp^{nsCre}$ transgene could be expressed in almost all AgRP

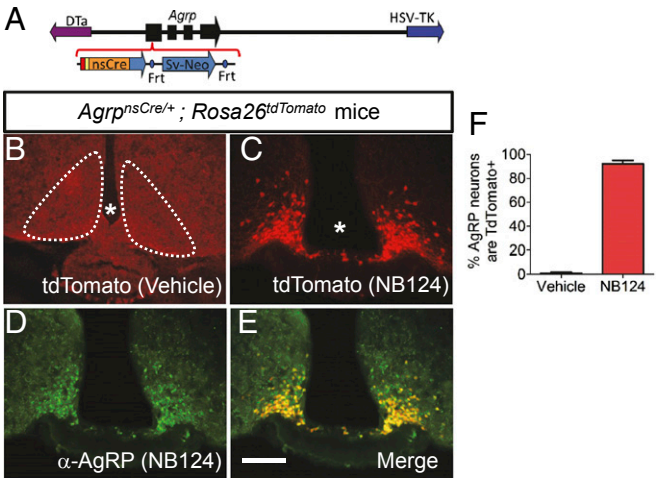

Fig. 2. Engineering an inducible $Agrp^{nsCre}$ transgenic mouse line. (*A*) A schematic diagram showing the targeting construct for generation of $Agrp^{nsCre}$ mice. Specifically, the *nsCre2*-containing cassette was cloned into *Agrp* gene locus immediately 5′ of the translation start codon. (*B*) Fluorescence image shows no leaky expression of tdTomato (red) in $Agrp^{nsCre/+}::Rosa26^{tdTomato}$ mice. Dotted circles indicate the ARC region of the hypothalamus where AgRP-expressing neurons are located. (*C*) Fluorescence image shows tdTomato expression profile (red) in the $Agrp^{nsCre/+}::Rosa26^{tdTomato}$ mice 4 d after injection of NB124 into the third ventricle. (*D*) Immunostaining image shows expression profile of AgRP (green) in the same mice as described in *C*. (*E*) Merged image of *C* and *D* illustrates the colocalization of tdTomato marker and AgRP peptides. (*F*) Quantification of AgRP neurons expressing tdTomato under vehicle or NB124 treatment. Asterisks in *B* and *C* indicate the third ventricle. (Scale bar: *B–E*, 300 μm.)

neurons (Fig. 2 *C–F*). The best regimen involved injecting 80 μg of NB124 twice (2 d apart); higher single doses revealed some toxic effects, including lethargy and dyspnea, and a single 80-μg dose did not result in complete recombination. These results demonstrate that this inducible gene targeting system can exhibit high efficiency and fidelity, but careful titration of the drug is necessary.

To demonstrate the usefulness of this new genetic approach in controlling an important physiological process, we set out to disrupt GABA synthesis by AgRP neurons, a small population of neurons in the ARC that is important for the control of feeding and energy metabolism (9, 24–27). We generated two different lines of mice for the behavioral assays: $Agrp^{nsCre/+}$::$Gad1^{lox/lox}$::$Gad2^{lox/lox}$::$Rosa26^{tdTomato}$ mice, termed the mutant group, and $Agrp^{nsCre/+}$::$Gad1^{lox/+}$::$Gad2^{lox/+}$::$Rosa26^{tdTomato}$ mice, termed the control group. These two groups of mice share identical genetic backgrounds, but carry either heterozygous or homozygous alleles of floxed *Gad1* or *Gad2*, genes encoding the two essential enzymes (GAD67 and GAD65) for GABA biosynthesis. Injection of NB124 into the third ventricle of the control group led to the expression of tdTomato in AgRP neurons, and GABA staining was retained (Fig. 3 *A–D*) (25, 28). In contrast, treatment with NB124 in the mutant group abolished GABA immunological detection exclusively in the tdTomato-labeled AgRP neurons (Fig. 3 *E–H*). To further demonstrate the efficacy of genetic inactivation, we quantitatively examined the expression level of *Gad1* and *Gad2* mRNA from AgRP neurons that were isolated by fluorescence-activated cell sorting (FACS). Real-time PCR results indicated that *Gad1* and *Gad2* mRNA in AgRP neurons were almost completely depleted in NB124-treated mutant mice compared to NB124-treated control or the $Agrp^{Cre}$::$Rosa26^{tdTomato}$ groups (Fig. 3 *I* and *J*). Furthermore, HPLC analysis revealed that the abundance of GABA in the ARC region of the NB124-treated mutant group was reduced to the same extent as displayed by diphtheria toxin (DT)-treated $Agrp^{DTR/+}$ mice, in which nearly all AgRP neurons were ablated (Fig. 3*K*) (29). The levels of glutamic acid and *Agrp* mRNA were unchanged in the ARC of the NB124-treated mutant group compared to wild-type or NB124-treated control mice (Fig. S3 *A* and *B*). In addition, strong Fos induction was observed in brain regions that receive afferents from AgRP neurons, including the bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus, and the parabrachial nucleus (PBN; Fig. S4 *A–F*) (25, 30). Deletion of *Gad1 and Gad2* from AgRP neurons did not affect GABA levels in other brain regions—including the lateral hypothalamic nucleus (LH), dorsal medial hypothalamic nucleus (DMH), and central amygdala (CeA)—suggesting high selectivity of the NB124-mediated, gene-knockout strategy (Fig. S5). Our results suggest that treatment with NB124 in the mutant mice leads to sufficient production of functional Cre recombinase to inactivate *Gad1* and *Gad2* specifically in the AgRP neurons within 4 d, thereby disinhibiting postsynaptic neurons resulting from the loss of GABA signaling from AgRP neurons. Note that during those 4 d, the *nsCre* gene had to be transcribed and translated; the *Gad*1 and *Gad2* alleles needed to recombine; *Gad1* and *Gad2* mRNAs and the two biosynthetic enzymes needed to decay; and GABA uptake from neighboring neurons had to be insufficient to maintain normal GABA signaling.

We characterized the effects on energy balance of NB124-mediated conditional deletion of GABA from AgRP neurons. The behavioral results revealed that injections of NB124 into the 3-mo-old mutant mice reduced food intake and resulted in significant loss of body weight (Fig. 4 *A* and *B*). Six days after initial treatment with NB124, these moribund mice had to be removed from the experiment because they had lost ∼20% of their initial body weight, and their daily food intake had almost ceased. In contrast, three groups of controls—including the control mice treated with NB124, control mice treated with vehicle, and

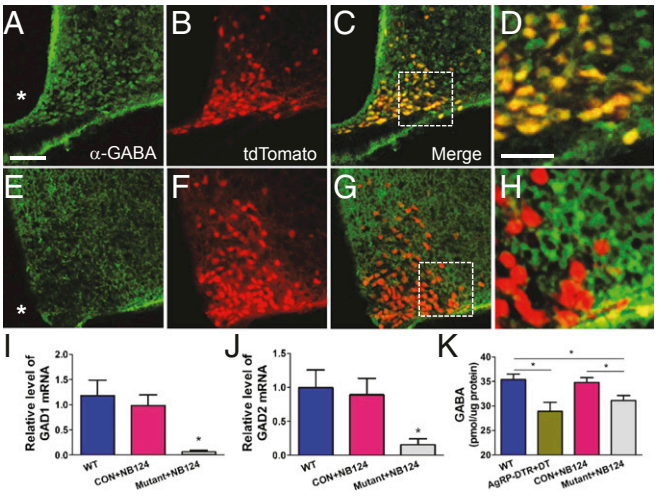

Fig. 3. Deletion of GABA neurotransmitter from the AgRP neurons upon NB124 treatment. (*A* and *B*) Immunostaining images show anti-GABA (*A*; green) and tdTomato (*B*; red) in the ARC of control group mice 4 d after NB124 treatment. (*C*) Merged image of *A* and *B* shows colocalization of GABA and tdTomato in the ARC. (*D*) High-magnification image of the dotted area in *C* shows that almost all AgRP neurons coexpress GABA neurotransmitter (yellow) in control mice. (*E* and *F*) Immunostaining images show anti-GABA (*E*) and tdTomato (*F*) in the ARC of mutant-group mice 4 d after NB124 treatment. (*G*) Merged image of *E* and *F* shows GABA and tdTomato in the ARC. (*H*) Higher-magnification image of the dotted area in *G* shows that almost all AgRP neurons are depleted of GABA in mutant mice. Asterisks in *A* and *E* indicate the third ventricle. (*I* and *J*) Real-time qPCR analysis of transcript levels of *Gad1* (*I*) and *Gad2* (*J*) expressed in AgRP neurons that were isolated by a flow cytometry approach from $Agrp^{Cre}$::$Rosa26^{tdTomato}$ mice (WT group) and control and mutant group mice treated with NB124. (*K*) Abundance of GABA in the ARC was measured by HPLC in wild-type mice, DT-treated $Agrp^{DTR/+}$ mice, and NB124-treated control and mutant groups. Values represent group means and SEM; $n = 5$ or 6 mice per group. $^*P < 0.05$ [analysis of variance (ANOVA) with SNK post hoc]. Control group, $Agrp^{nsCre}$::$Rosa26^{tdTomato}$ mice; mutant group, $Agrp^{nsCre/+}$::$Gad1^{lox/lox}$::$Gad2^{lox/lox}$::$Rosa26^{tdTomato}$ mice. (Scale bars: *A–C* and *E–G*, 100 μm; *D* and *H*, 60 μm.)

mutant mice treated with vehicle—showed normal daily food intake and body weight throughout the testing period (Fig. 4 *A* and *B*). To assess the potential role of GABA in mediating energy expenditure, we measured metabolic responses in Comprehensive Lab Animal Monitoring System chambers (Columbus Instruments). In comparison with the control groups, the mutant group showed significant increases in O_2 consumption, CO_2 production, and locomotor activity between 2 and 4 d after the first treatment with NB124 (Fig. 4 *C–E*). Furthermore, loss of GABA from AgRP neurons resulted in glucose intolerance, compared with both the NB124-treated control mice and vehicle-treated mutant mice that were pair-fed to the same degree of weight loss (Fig. 4*F*). In line with a recent study, these results provide direct evidence that GABA signaling by AgRP neurons normally promotes food intake while simultaneously inhibiting energy expenditure (31). More surprisingly, this experiment revealed that GABAergic signaling by AgRP neurons also plays a role in maintaining normal glucose metabolism.

We also tested the role of GABA signaling from AgRP neurons in the control of feeding and metabolism in 8-mo-old mice because of reports of synaptic remodeling by aging AgRP neurons (32). Compared with the age-matched control group, i.c.v. delivery of NB124 into 8-mo-old mutant mice activated Cre-dependent tdTomato reporter gene and reduced *Gad1* and *Gad2* transcript levels by ∼90% in AgRP neurons within 6 d (Fig. S6). Surprisingly, deletion of GABA synthesis from AgRP neurons in older mice mediated a moderate, but only transient, reduction of food intake and body weight (Fig. 5 *A* and *B*). Furthermore, no

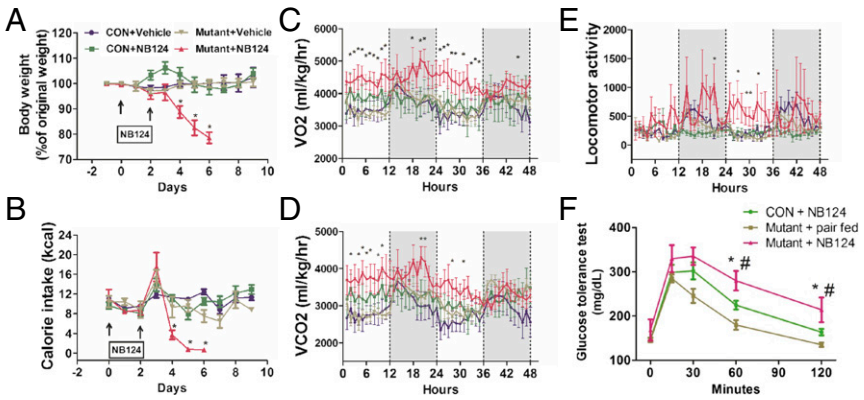

Fig. 4. Acute inactivation of GABA signaling from AgRP neurons in 3-mo-old adult mice leads to reduction of feeding, severe weight loss, enhanced energy expenditure, and glucose intolerance. (*A* and *B*) Body weight (*A*) and daily calorie intake (*B*) of the following four groups of mice with drug or vehicle delivered into the third ventricle: control mice treated with vehicle, control mice treated with NB124 (80 μg × 2, i.c.v.), mutant mice treated with vehicle, and mutant mice treated with NB124 (80 μg × 2, i.c.v.). (*C*–*E*) Immediately after the second treatment of NB124, O_2 consumption (*C*), CO_2 production (*D*), and locomotor activity (*E*) of the mice as described in *A* and *B*. $^*P < 0.05$ (between mutant+NB124 group and the control groups; ANOVA with SNK post hoc). (*F*) Glucose tolerance test (GTT) was performed in control mice treated with NB124, mutant mice pair-fed to the mutant group treated with NB124, and mutant group treated with NB124. $^*P < 0.05$ (between mutant+NB124 group and control+NB124 group; Student *t* test); $^{\#}P < 0.05$ (between mutant+NB124 group and mutant+pair-fed group; Student *t* test). Values represent group means and SEM; $n = 8$–10 mice per group. Control group, *Agrp*$^{nsCre/+}$::*Gad1*$^{lox/+}$::*Gad2*$^{lox/+}$::*Rosa26*^{tdTomato} mice; mutant group, *Agrp*$^{nsCre/+}$::*Gad1*$^{lox/lox}$::*Gad2*$^{lox/lox}$::*Rosa26*^{tdTomato} mice.

significant changes were observed in energy metabolism, locomotion, or glucose metabolism (Fig. 5 *C*–*F*). Notably, deletion of GABA from AgRP neurons in 8-mo-old mice resulted in Fos induction in postsynaptic neurons, but to a lesser extent compared with young mice (Fig. S4). These data suggest that AgRP neurons in older mice are more resistant to the loss of GABA signaling.

Discussion

In this study, we established a new method for inducible Cre-dependent inactivation of *loxP*-flanked genes and demonstrated its effectiveness by eliminating GABA signaling from AgRP neurons and monitoring biological consequences on behavior and metabolism. Abrupt inactivation of GABA biosynthesis genes in adult mice permitted us to interrogate the pathophysiological roles exhibited by this key inhibitory neurotransmitter in AgRP neurons at various ages. We chose this model system because constitutive inactivation of GABA signaling starting when *Agrp* genes become active during late embryonic development results in a compensatory response, such that there is minimal effect on food intake or body weight of adult mice, a response that is similar to that observed when AgRP neuron are ablated in newborn mice (9, 29). In contrast, ablation of AgRP neurons or disruption of GABA signaling in young adult mice leads to anorexia and severe weight loss (29, 33, 34). Considerable evidence indicates that this starvation phenotype is mediated by hyperactivity of neurons that express calcitonin gene-related protein in the PBN due to loss of GABA signaling from AgRP neurons (35). We discovered that chronic infusion of a partial $GABA_A$ agonist for 2 wk can prevent starvation after ablation of AgRP neurons, and, importantly, food intake and body weight were maintained even after termination of $GABA_A$ agonist infusion (27), suggesting that mice had adapted to loss of AgRP neurons (28). Mice can also adapt if the ablation is performed in moderately obese, leptin-deficient mice (36), presumably because they have sufficient energy reserves to sustain them while

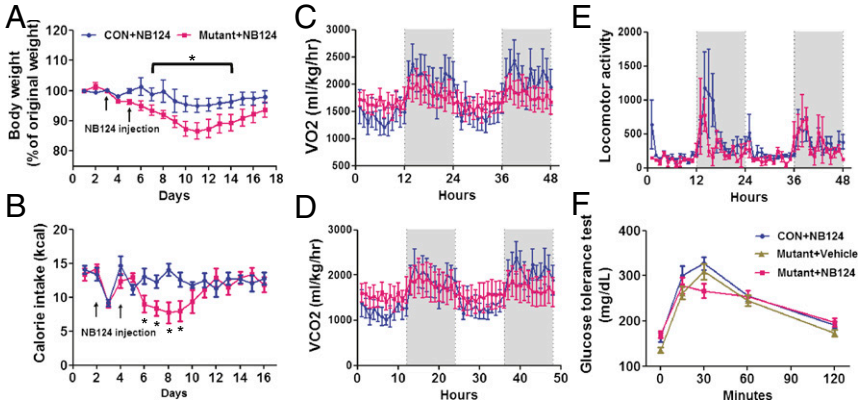

Fig. 5. Metabolic characterization of 8-mo-old mice upon acute disruption of GABA signaling from AgRP neurons. (*A* and *B*) Body weight (*A*) and daily calorie intake (*B*) of the following two groups of mice with drug or vehicle delivered into the third ventricle: control mice treated with NB124 and mutant mice treated with NB124 (80 μg × 2, i.c.v.). (*C*–*E*) Immediately after the second treatment of NB124, O_2 consumption (*C*), CO_2 production (*D*), and locomotor activity (*E*) of the mice as described in *A* and *B*. (*F*) GTT was performed in control mice treated with NB124, mutant mice treated with vehicle, and mutant group treated with NB124. $^*P < 0.05$ (between mutant+NB124 group and control+NB124 group; Student *t* test). Values represent group means and SEM; $n = 8$–10 mice per group. Control group, *Agrp*$^{nsCre/+}$::*Gad1*$^{lox/+}$::*Gad2*$^{lox/+}$::*Rosa26*^{tdTomato} mice; mutant group, *Agrp*$^{nsCre/+}$::*Gad1*$^{lox/lox}$::*Gad2*$^{lox/lox}$::*Rosa26*^{tdTomato} mice.

adaptation occurs. We suggest that loss of GABA signaling by AgRP neurons in young mice triggers a similar compensatory mechanism that can supersede the physiological function of these neurons. Apparently, NPY and AgRP alone are unable to sustain adequate food intake when only GABA is acutely eliminated from AgRP neurons in young mice. Conversely, we suggest that ectopic enhancement of GABA input and/or $GABA_A$ receptor signaling at postsynaptic targets of AgRP neurons could be critically involved in the compensatory mechanism.

Previously, AgRP neuron ablation experiments were performed in relatively young mice. Thus, we were surprised that the 8-mo-old mice in this study were resistant to the severe consequences of inactivating GABA signaling, suggesting that there is an age-related adaptive mechanism that allows adequate feeding in the absence of AgRP neurons. We suggest that long-term nutritional or environmental conditions favor desensitization of homeostatic feeding mediated by AgRP neurons, while promoting reliance on the brainstem feeding circuit and/or midbrain reward circuit (37). These ancillary feeding pathways may progressively overtake the physiological role of AgRP neurons under an emergent or age-dependent mechanism. The reduced Fos induction in postsynaptic targets of the older mice further suggests that an age-dependent mechanism promotes the establishment of a novel balance between excitatory and inhibitory inputs onto these brain regions, where GABA from AgRP neurons has less impact upon the excitability of postsynaptic neurons, such as the calcitonin gene-related peptide neurons in the PBN (28, 35).

In contrast to the Cre^{ER} and Cre^{PR} systems, we avoided the problem of tethering Cre recombinase with a bulky addition by introducing a single amino acid into a particular locus within Cre recombinase—a strategy that prevents gene silencing, at least at the *Agrp* locus. Aminoglycoside derivatives, unlike lipid-soluble tamoxifen and RU486, cannot penetrate the blood–brain barrier or diffuse far from the site of injection (38). Thus, the *nsCre* system is particularly useful to distinguish between central and peripheral contributions when the gene of interest is expressed in both periphery and brain, such as the *Rip–Cre* case (3, 4). Furthermore, one might be able to target one brain region with an aminoglycoside without affecting genes in others regions. Some studies indicated that tamoxifen and RU486 interfere with reproductive and heart physiology, which could affect interpretation of acquired phenotypes (39, 40). Moreover, emerging evidence indicates that constitutive expression of full-length functional Cre or Cre^{ER} protein exhibits serious metabolic side effects, such as *Nestin–Cre* on pituitary functions as well as *Rip–Cre or* Cre^{ER} on β-cell functions (3, 41). In contrast, PTC promotes NMD, a conserved pathway that destroys the majority of *nsCre* transcripts, thereby leaving only a few mutant mRNAs that are translated into truncated peptides (13). Furthermore, aminoglycosides have not been cited for causing adverse effects on fertility, heart physiology, digestive systems, or central nervous systems (14, 16). Although aminoglycosides generally elicit nonspecific cytotoxicity, the dosage of a new class of G418 derivatives, including NB124, that we used did not reveal noticeable adverse effects on feeding, body weight, locomotion, or energy metabolism in the control group. We used aminoglycoside activation of *nsCre* to both activate a reporter gene ($Rosa26^{tdTomato}$) and inactivate *Gad1* and *Gad2* genes, thus demonstrating two useful applications. This nonsense suppression-based genetic system provides an ideal alternative strategy for regulating expression of Cre-dependent genes in diverse scenarios where other inducible Cre strategies are either nonapplicable or problematic.

Materials and Methods

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine, the University of Iowa, and the University of Washington. To generate *nsCre* transgene, a nonsense codon, UGA, was introduced into P1 phage Cre recombinase coding sequence by a PCR-based replacement method. The inducible $Agrp^{nsCre}$ and the conditional $Gad2^{lox/+}$ transgenic lines were generated by standard cloning and ES cell-based gene targeting protocols. Other mouse strains have been reported (22, 25, 29, 42). Mice were microinjected with 0.8 μL of G418, NB compounds, or saline vehicle into the third ventricle either once or twice (2 d apart). Measurements of GABA and glutamate concentrations, immunohistochemistry, quantitative PCR, and FACS techniques were performed by standard procedures (36, 43, 44). Food intake, body weight, energy expenditure, and glucose tolerance test (GTT) were measured as described (28, 36, 45). All statistical analyses were performed by Microsoft Excel and GraphPad Prism. Refer to *SI Materials and Methods* for experimental details.

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