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Nrf2 Gene Deletion Fails to Alter Psychostimulant-induced Behavior or Neurotoxicity

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Abstract

The transcription factor NF-E2-related factor (Nrf2) regulates the induction of phase 2 detoxifying enzymes by oxidative stress, including synthesis of the catalytic subunit (xCT) of the heterodimeric cystine-glutamate exchanger (system xc-). Repeated cocaine treatment in rats causes persistent neuroadaptations in glutamate neurotransmission in the nucleus accumbens that result, in part, from reduced activity of system xc-. Since in vitro under- or over-expression of Nrf2 regulates system xc- activity and xCT content, it was hypothesized that in vivo deletion of the Nrf2 gene would: 1) decrease system xc- activity, 2) produce a behavioral phenotype resembling that elicited by chronic cocaine administration, and 3) enhance dopamine depletion after methamphetamine-induced oxidative stress. In all three experiments no genotypic difference was measured between mice sustaining homozygous Nrf2 gene deletion and wild-type littermates. Thus, while Nrf2 is a transcriptional regulator of xCT and capable of protecting cells from oxidative stress, following Nrf2 gene deletion this role can be partially compensated by other mechanisms and methamphetamine-induced oxidative stress and dopamine toxicity does not significantly involve Nrf2.

Keywords

Nrf2; knockout mice; cocaine; glutamate; dopamine; methamphetamine; system xc-

1. Introduction

Repeated cocaine treatment produces enduring behavioral and neurochemical changes that arise, in part, from cocaine-induced plasticity in glutamate neurotransmission in the nucleus accumbens, a brain region critically involved in cocaine's addictive properties (Kalivas et al., 2005;Wolf,1998). Among the long-term changes shown to be associated with cocaine-seeking in self-administration and reinstatement paradigms is an increase in synaptic glutamate release and a reduction in basal nonsynaptic extracellular levels of glutamate (Baker et al., 2003;McFarland et al.,2003). In the accumbens of rats, the basal extracellular level of glutamate is maintained by the cystine/glutamate exchanger (system xc-) (Baker et al.,2002), which

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promotes the substrate-dependent exchange of one extracellular cystine for one intracellular glutamate and is the rate-limiting step in synthesis of the intracellular antioxidant glutathione (McBean,2002). Repeated cocaine treatment in rats reduces the affinity of the cystine/glutamate exchanger leading to lowered basal extracellular levels of glutamate in the accumbens and disinhibition of synaptic glutamate release (Baker et al.,2003;Moran et al., 2005). The restoration of system xc- by cysteine pro-drugs prevents the reduction in basal glutamate levels in the accumbens, as well as cocaine-seeking in the reinstatement model of relapse (Baker et al.,2003).

System xc- is a heterodimeric protein complex consisting of a catalytic chain (xCT), unique to the exchanger, and a heavy chain (4F2hc) common to many amino acid transporters and probably important for membrane localization (Bassi et al.,2001;Sato et al.,1999;Shih and Murphy,2001). The expression of the gene encoding xCT is controlled, in part, by a transcription factor called NF-E2 related factor 2 (Nrf2) (Sasaki et al.,2002). Nrf2 is the major transcriptional regulator of phase 2 detoxifying enzymes by binding the antioxidant responsive element (ARE) (Itoh et al.,1997;Sasaki et al.,2002). Oxidative stress regulates Nrf2 transcriptional activity by controlling its intracellular distribution. Under basal conditions, Nrf2 is bound to Keap1 (Kelch-like ECH associating protein 1) and sequestered in the cytoplasm where it is targeted for ubiquitin-mediated degradation (Itoh et al.,1999;Katoh et al.,2005). Nrf2 and Keap1 exist as a complex that can move between the nucleus and the cytoplasm. In response to oxidative stress the Nrf2-Keap1 complex accumulates in the nucleus, where it dissociates leaving Nrf2 free to dimerize with Maf proteins to form a transcriptional activation complex capable of binding the ARE promotor region and increasing the expression of xCT and other phase 2 enzymes (Dinkova-Kostova et al.,2005;Itoh et al.,1997;Katsuoka et al., 2005).

Nrf2 gene deletion prevents the induction of cystine uptake produced by agents inducing oxidative stress (e.g. diethyl maleate, paraquat, $CdCl_2$) in embryonic fibroblast (Sasaki et al., 2002) and macrophage cultures (Ishii et al.,2000). Also, in primary neuronal or glial cultures the loss of Nrf2 reduces the constitutive and inducible expression of cytoprotective genes (Lee et al.,2003a;Lee et al.,2003b;Shih et al.,2005), and Nrf2 over-expression in glia cells protects neurons from oxidative stress (Shih et al.,2003). Finally, constitutive deletion of the Nrf2 gene in vivo reduces the induction of glutathione-dependent enzymes in the liver, and augments cortical and striatal lesions induced by the mitochondrial complex II inhibitor 3-nitropropionic acid (Calkins et al.,2005;Chanas et al.,2002;Shih et al.,2005). Therefore, it has been concluded from in vitro and in vivo studies that Nrf2 controls the expression of xCT as part of a cellular response that protects cells against oxidative stress.

Based upon the predominantly in vitro data showing a role for Nrf2 in regulating xCT expression and the in vivo role of system xc- in the enduring behavioral and neurochemical effects of repeated cocaine we tested three hypotheses. 1) Homozygous deletion of the Nrf2 gene in mice (Nrf2 KO) will decrease system xc- basal activity by reducing xCT expression. 2) Nrf2 KO will show alterations in cocaine-induced locomotion, locomotor sensitization and conditioned place preference compared to wild-type littermates (WT). 3) Nrf2 KO will be more susceptible to methamphetamine(METH)-induced neurotoxicity. This latter hypothesis was based on evidence that METH-induced toxicity to dopamine (DA) terminals results, at least in part, from the induction of reactive oxygen species (ROS), such as superoxides and free hydroxyradicals (Pubill et al.,2005), that ROS promote Nrf2 induction of detoxifying enzymes (Kita et al.,2003), and that treatment with antioxidants attenuates METH toxicity (Hashimoto et al.,2004).

2. Results

2.1. Nrf2 KO and WT mice have equivalent basal system xc- activity and levels of xCT

Figure 1A shows that in ventral striatal slices (containing nucleus accumbens), homozygous Nrf2 deletion did not alter system xc- basal activity as measured by Na^+-independent L-[^{3}H]-glutamate uptake in pmoles/mg protein/minute. A two-way ANOVA using Genotype and L-Glutamate concentration as main factors, showed a significant effect of L-glutamate concentration ($F(2,16)= 133.56$, $p<0.0001$) but no genotypic differences between Nrf2 KO and WT. Consistent with the apparent lack of effect by Nrf2 deletion on system xc- activity, immunoblotting for xCT, the catalytic subunit of system xc-, revealed no differences in xCT expression in ventral or dorsal striatum, or in the Prefrontal Cortex (PFC) between Nrf2 KO and WT mice (figure 1B and C). The lack of genotypic difference in xCT levels in the striatum and PFC is in contrast to the meninges, where xCT is highly concentrated (Sato et al.,2002), and Nrf2 deletion caused a nearly 50% reduction in xCT (Shih et al.,2006b).

2.2. Nrf2 deletion did not modify cocaine-induced place conditioning or locomotor sensitization

Figure 2A, shows the time spent in the drug-paired side during Pre- and Post-Test trials in the place conditioning paradigm, and reveals that both Nrf2 KO and WT mice displayed an equivalent increase in the time spent in the cocaine-paired side. A threeway ANOVA with repeated measures over Test, using Drug (saline and cocaine) and Genotype as between subjects factors showed that cocaine (10mg/kg, i.p.) induced place conditioning in both Nrf2 KO and WT mice [Post vs Pre-Test $F(1,44)= 34$, $p< .0001$; Post vs Pre-Test x Drug interaction $F(1,44)= 8.76$, $p< .005$].

Figure 2B, shows the total locomotor activity (in photocell beam breaks) during each 15 min conditioning session on the drug-paired side in the place conditioning experiment shown in figure 2A. The data reveal the acute locomotor stimulant effect of cocaine, and the sensitization of the acute effect following repeated treatment. Akin to place conditioning, there was no genotypic difference in the locomotor response to cocaine. A three-way ANOVA with repeated measures over conditioning session showed that Nrf2 KO and WT mice exhibited cocaine-induced locomotor stimulant effect [Drug x sessions interaction $F(3,132)= 4.09$, $p< 0.02$], and showed sensitization of the cocaine stimulant effect with daily injection [first vs last session $F(1,44)= 16.2$, $p<0.0001$]. However, if the data containing the first injection of saline or cocaine (S1) was isolated and evaluated using a two-way ANOVA, a significant effect was measured for both genotype ($F(1,29)= 19.28$, $p< 0.0001$) and drug treatment ($F(1,29)= 5.71$, $p< 0.03$). To further investigate the possibility that there was an effect of Nrf2 gene deletion on the acute locomotor response to cocaine, separate animals were administered saline or cocaine (3 or 10 mg/kg, i.p.), separated by a one week intertrial interval. Figure 2C shows that while there was a significant effect of cocaine ($F(2,20)= 6.80$, $p< 0.006$), there was no effect of genotype or interaction between genotype and cocaine.

2.3. No genotypic differences in METH-induced locomotion, DA depletion or system xc- activity

METH (5.0 or 10.0 mg/kg, i.p.) was administered 4 times, and total distance was measured during 20 min after the first and the last injection (Figure 3A). Acute METH produces an increase in locomotion by releasing DA, and binge METH administration (4 injection, every 2 hours) is known to deplete DA in synaptic terminals and decrease locomotion (Seiden et al., 1993). Correspondingly, figure 3A shows an acute motor stimulant effect and a reduction in locomotor activity when comparing the first to last binge injection of METH. A three-way ANOVA with repeated measures over METH injection showed that the total distance traveled in response to the first injection was higher than the response to the last injection ($F(1,53)=$

110.89, p<0.001). Also, the METH response was significantly higher than Saline (main effect Drug F(2,53)= 10.54, p<0.001), and there was an interaction between drug dose and injection number (F(2,53)= 20.838, p<0.001). There was no significant effect or interaction involving genotype as a factor. Stereotypy counts were also analyzed and the results showed the same pattern as distance traveled (data not shown). A three-way ANOVA revealed no significant effect or interaction with genotype, but a significant main effect of injection number (F(1,53) = 60.28, p< 0.0001) and drug dose (F(2,53)= 20.01, p< 0.0001) was observed.

Two weeks after discontinuing METH administration DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured in the ventral striatum, dorsal striatum and PFC. Table 1 shows that there was significant depletion of DA by both doses of METH in the ventral and dorsal striatum. However, there was no difference between Nrf2 KO and WT littermates in the extent of DA depletion. A three-way ANOVA revealed a significant main effect of drug dose (ventral striatum- F(2,39)=7.88, p< 0.005; dorsal striatum- F(2,42)=41.25, p< 0.0001). Post hoc comparisons showed that both METH doses reduced DA levels compared with saline, and in the dorsal striatum the highest dose of METH produced a greater DA reduction than the lowest dose. Similar effects were also seen with DA metabolites, except that significant effects were measured only at the highest dose of METH for DOPAC and HVA in the dorsal striatum and for DOPAC in the ventral striatum, while METH did not significantly alter HVA levels in the ventral striatum (Table 1). As with DA, no genotypic effects were measured in levels of DA metabolites. In contrast to the striatal regions, METH had no effect on DA or its metabolites in the PFC (data not shown). Similar to no apparent interaction between Nrf2 and METH-induced DA depletion, figure 3B shows that two weeks after discontinuing repeated METH (10 mg/kg, ip) system xc- activity in ventral striatal slices was equivalent between Nrf2 KO and WT littermates. While a twoway ANOVA did not reveal a significant effect of genotype on glutamate uptake, there was a significant effect of glutamate concentration (F(2,8)= 218.83, p<0.0001).

3. Discussion

The data show that constitutive homozygous deletion of the Nrf2 gene did not affect baseline activity of system xc- or protein levels of xCT in the ventral striatum. Correspondingly, Nrf2 deletion did not modify cocaine-induced behavioral effects either after acute or repeated administration, which have been shown to be associated with reduced system xc- activity in rats (Baker et al.,2003). Furthermore, Nrf2 deletion did not alter vulnerability to METH-induced DA depletion.

The lack of interaction between Nrf2 gene deletion and psychostimulants was not predicted based upon the well-studied regulation of xCT synthesis by the Nrf2-Maf transcriptional complex and the capacity of chronic cocaine to regulate system xc- activity in rats. Moreover, Nrf2 induces synthesis of a battery of enzymes that coordinate a protective response to oxidative stress via binding to the ARE promoter region, which is present in the xCT gene (Chanas et al.,2002;Sasaki et al.,2002;Shih et al.,2005). Much of the previous research using Nrf2 KO has been conducted in cultured embryonic cells, and these studies clearly show that cells lacking Nrf2 are deficient in the induction of phase 2 antioxidant enzymes by oxidative stressors (Ishii et al.,2000;Kraft et al.,2004;Lee et al.,2003a;Lee et al.,2003b). While one of these studies showed a reduction in the ability of toxic electrophiles to induce system xc- activity in macrophages cultured from Nrf2 KO (Ishii et al.,2000), studies employing Nrf2 KO cultured glia or neurons showed increased sensitivity to oxidative toxicity, but did not identify a specific role for deficits in system xc- or xCT expression (Kraft et al.,2004;Lee et al., 2003a;Lee et al.,2003b). To our knowledge, twelve in vivo studies with Nrf2 KO have previously examined toxicity to oxidative stress. Four studies have shown deficient hepatic induction of glutathione-dependent enzymes (specifically glutathione-S-transferases) by

butylated hydroxyanisole in Nrf2 KO, but did not assay system xc- or xCT (Chan and Kwong, 2000;Chanas et al.,2002;Hayes et al.,2000;Satoh et al.,2002). Four other studies showed vulnerability to hepatic toxicity after treatment with acetaminophen or pentachlorophenol (Chan et al.,2001;Enomoto et al.,2001;Okawa et al.,2006;Umemura et al.,2006). Also, the induction of glutathione-S-transferases and γ-glutamylcysteine synthetase (synthetic enzyme in glutathione synthesis) in the small intestine after treatment with sulforaphane or other chemoprotective agents was absent in Nrf2 KO (Hayes and McMahon,2001;Thimmulappa et al.,2002). In more recent papers, Nrf2 KO were shown to be hypersensitive to 3-nitropropionic acid and malonate induced lesions in the striatum(Calkins et al.,2005;Shih et al.,2005), and more susceptible to seizures, neuronal damage and microglial infiltration in hippocampus induced by kainic acid exposure (Kraft et al.,2006). In all cases toxicity was presumably a result of deficient induction of phase 2 antioxidant enzymes.

In light of the existing in vitro and in vivo studies with Nrf2 KO, how do we explain the negative data in the present report? Perhaps most confusing is the lack of effect by Nrf2 gene deletion on system xc- activity and xCT protein expression in striatal tissue. While data from in vitro studies over-expressing Nrf2 clearly demonstrate that Nrf2 regulates xCT synthesis and system xc- activity (Shih et al.,2005;Shih et al.,2003), Nrf2 may not control basal xCT expression in most brain tissues. Moreover, important caveats exist in the only study with Nrf2 KO that found deficient induction of system xc- by oxidative stress. For example, the induction of system xc- was not abolished by Nrf2 deletion, basal system xc- activity was equivalent between Nrf2 KO and WT, and the induction of system xc- by lipopolysaccharide was intact in Nrf2 KO (Ishii et al.,2000). Finally, recent evaluation of xCT levels in many brain regions replicated the lack of effect in striatum after deletion of the Nrf2 gene, but found a modest reduction in parietal cortex, and a nearly 50% reduction in the meninges where xCT levels are particularly high (unpublished observations, Andy Shih and Timothy Murphy, University of British Columbia; (Sato et al.,2002). Of note, however, substantial xCT protein remained in all brain regions of the Nrf2 KO. Taken together, these data indicate that while Nrf2 can regulate xCT synthesis and thereby system xc- activity, this regulation is not obligatory and other regulatory factors can control xCT synthesis, perhaps more so when the Nrf2 gene has been deleted. Examples of other transcription factors that can promote the expression of phase 2 antioxidant enzymes include Nrf1, Jun-B and Jun-D (Jaiswal,2000;Myhrstad et al.,2001).

The role of system xc- in regulating cocaine induced drug-seeking in rats was shown by the down-regulation of system xc- activity after chronic cocaine administration thereby causing a significant reduction in basal levels of extracellular glutamate in the accumbens (Baker et al., 2003). Moreover, system xc- has been shown to regulate synaptic release of glutamate by maintaining glutamatergic tone on inhibitory metabotropic glutamate autoreceptors (Moran et al.,2005), and this appears to contribute to the marked increase in synaptic glutamate release in the accumbens of rats produced during drug-seeking, as well as during the expression of locomotor sensitization (Baker et al.,2003;Pierce et al.,1996). It is important to note here that while cocaine's effect on system xc- activity and expression have been studied in rats, there is less evidence in mice. The primary extant data supporting involvement of xc- in mice is a study showing that deletion of the Homer2 gene results in a behavioral phenotype resembling pre-sensitization to cocaine (i.e. Homer2 KO mice show enhanced conditioned place preference, locomotor stimulation and acquisition of self-administered cocaine compared to WT mice) (Szumlinski et al., 2004). Accordingly, Homer2 deletion in drug naïve Homer2 KO mice was associated with reduced system xc-, akin to that produced by chronic cocaine treatment in rats. Taking all of these studies together, had the present study found Nrf2 to be a potent positive regulator of system xc- activity or xCT levels in the ventral striatum (including nucleus accumbens), it would be predicted that Nrf2 KO might show altered behavioral responding to cocaine. However, the lack of genotypic difference in cocaine-induced place conditioning or

locomotor stimulation is consistent with an apparent lack of difference in system xc- activity or xCT protein.

Finally, even if Nrf2 gene deletion did not impact cocaine-induced behaviors, presumably by not mimicking the cocaine-induced reduction in system xc- activity, the fact that all studies to date clearly show that other phase 2 antioxidant enzymes are potently regulated by Nrf2 one would predict that the loss of Nrf2 would render mice more sensitive to the DA depleting effects of METH. Indeed, DA depleting METH treatments induce ROS (Pubill et al.,2005), ROS promote Nrf2 induction of detoxifying enzymes, and treatment with antioxidants attenuate methamphetamine toxicity (Hashimoto et al.,2004;McCann and Ricaurte,2004). Also, Nrf2 KO have been shown to be hypersensitive to in vivo neurotoxicity induced by other oxidative stressors, such as the mitochondrial complex II inhibitor 3-nitropropionic acid or *tert*-butylhydroquinone (Calkins et al.,2005;Chanas et al.,2002;Shih et al.,2005). Since agents well established to induce Nrf2 and phase 2 antioxidant enzymes are substantially more toxic in the absence of Nrf2, it may be that METH-induced DA depletion is not a strong enough stimulus to activate Nrf2. Consistent with this possibility, DA release by METH is essential to METH-induced DA depletion (McCann and Ricaurte,2004), and exposure of cultured astrocytes or meningeal cells to extracellular DA induces Nrf2 transcription (Shih et al.,2006a). However, the threshold for significant induction of Nrf2 by DA in vitro was 30 μM for 24 hrs (Shih et al.,2006a), and the maximum levels of extracellular DA induced by an in vivo METH regimen akin to that used in the present study have been reported to be between 0.1 to 1 μM (Holson et al.,1996;Stephans and Yamamoto,1994). Thus, the levels of extracellular DA achieved after DA-depleting doses of METH may not be sufficient to induce Nrf2, accounting for the lack of enhanced METH-induced DA depletion in Nrf2 KO.

In conclusion, homozygous deletion of the Nrf2 gene in mice did not affect system xc- baseline activity, cocaine-induced behaviors or METH-induced DA depletion. Thus, in contrast to predictions derived primarily from in vitro data showing regulation of system xc- by Nrf2, when the Nrf2 gene was constitutively deleted in vivo there was no measurable effect on system xc- activity or xCT protein in striatum. Moreover, since Nrf2 gene deletion did not alter METH-induced striatal DA depletion, indicating that ROS induction by METH does not significantly activate Nrf2 cytoprotective mechanisms.

4. Experimental Procedures

4.1. Experimental Subjects

Nrf2 KO adult mice (8-10 weeks age; 129sv × C57BL/6 background) originated from the laboratory of Dr. Yuet Wai Kan (Chan et al.,1996). Mice bred from heterozygous mating pairs resulted in WT, KO and heterozygous mice in the litter. At the age of 4 weeks, each mouse was genotyped. DNA was isolated from their tails and amplified by PCR using the following primers; primer 1 5′ GGAATGGAAAATAGCTCCTGCC 3′, primer 2 5′ GCCTGAGAGCTGTAGGCCC 3′, primer 3 5′ GGGTTTTCCCAGTCACGAC 3′. PCR conditions were as follows; one cycle at 95°C for 5 min, followed by 35 cycles at 95°C for 60s, 56°C for 60s, 72°C for 60s. Samples were then heated to 72°C for 8 min and then cooled to 4°C until being run on a 1.5% agarose gel. KO mice showed a single band at 214bp. WT mice showed a band a 262bp. Heterozygous mice showed both bands. All genotypic comparisons were between KO and their respective WT littermates. At least one week before testing mice were housed individually in an AAALAC-approved animal facility (lights on, 08:00 hr; 25°C). All testing was conducted during the light cycle. Food and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and conducted in accordance with the National Institutes of Health (NIH) *Principles of Laboratory Animal Care* (1985). Both male

and female mice were employed and for final analysis the data were pooled between genders because no significant gender effect was measured in any data set.

4.2. Place Conditioning and Cocaine-Induced Locomotion

Cocaine reward was assessed using a place conditioning procedure and apparatus similar to that described previously (Szumlinski et al.,2004). Briefly, place conditioning proceeded in the following four sequential phases: habituation, pre conditioning test (Pre-Test), conditioning, and post conditioning test (Post-Test). Each test lasted for 15 min. During the Habituation, Pre-Test and Post-Test the mouse was allowed to explore both compartments of the apparatus. The time spent in each compartment during the Pre-Test was recorded to establish compartment preferences. Cocaine conditioning was conducted over an 8 days period. Mice were brought into the conditioning room and were injected (0.01 ml/g body weight, i.p.) with cocaine (10 mg/kg, i.p.; kindly provided by NIDA, Bethesda, MD) and placed in the conditioning chamber. Mice received a saline injection in their preferred compartment and a cocaine injection in the non-preferred compartment. Control groups received saline in both compartments. The photocell counts in the cocaine-paired compartment during conditioning provided an index of cocaine-induced locomotion. For place conditioning studies, a Post-Test was conducted the day following the last conditioning session.

An additional experiment was conducted to evaluate the acute locomotor effect of cocaine in which locomotor activity was recorded for 60 min after cocaine or saline administration using a photocell apparatus that contained 8 photocells to record movement and software to estimate distance traveled by the animal based on consecutive breaking of adjacent photobeams vs breaking of the same photobeam (plexiglas activity chambers; 22 × 43 × 33 cm; Accuscan, Columbus, OH). Each animal received all three injections (saline, and cocaine at 3 and 10 mg/kg, ip) separated by a minimum 7 days intertrial interval.

4.3. METH treatment and biogenic amines measurement in brain tissue

Four METH injections separated by two hours, produces neurotoxicity in dorsal striatum in mice as measured by depletion of tissue DA levels (Cooney et al.,1998). To evaluate whether Nrf2 KO mice were more vulnerable to METH-induced neurotoxicity, the Nrf2 KO and WT mice were randomly assigned to one of three conditions (saline, METH: 5 and 10 mg/kg, i.p.; (+) methamphetamine HCl, Sigma/RBI, St Louis, MO), and received 4 injections of saline or METH. Locomotor activity after the first and last METH injection was monitored in for 20 min using the aforementioned photocell apparatus (Accuscan).

Two weeks after METH treatment, the mice were decapitated and the ventral and dorsal striatum, and the PFC were dissected from the brain as described elsewhere (Szumlinski et al., 2004), and immediately frozen on dry ice and kept in a −80°C freezer until analysis. Bilateral tissue samples were placed in 300 μl of mobile phase containing 0.2 μM isoproterenol as an internal standard, sonicated and centrifuged (2 min at 13000 rpm). The protein content in the resulting pellet was measured using the Bradford assay (Pierce). The concentration of DA and its metabolites DOPAC and HVA were quantified by injecting 5μl of supernatant into an HPLC with electrochemical detection. An ESA pump (model 582, ESA, Chelmsford, MA, USA) was used to deliver 0.5 ml/min of mobile phase (0.1 M trichloroacetic acid, 0.01 M sodium acetate, 0.1 mM EDTA and 16% MetOH. pH= 4.1) to a reversed phase column (ESA HR-80). After separation on the column, the biogenic amines were reduced/oxidized using coulometric detection (Coulochem II; ESA Inc.). Three electrodes were used: a guard cell (+0.40 V), a reduction analytical electrode (E1, −0.15 V), and an oxidation analytical electrode (E2, +0.325 V). The area under the peaks was measured using ESA 501 Chromatography Data System and the values were compared with an external standard curve for quantification. The final data were expressed as picomoles per milligram of protein.

4.4. L-[^{3}H]-Glutamate uptake in ventral striatal slices

The transport activity of system xc- can be monitored via Na^+-independent uptake of [^{35}S]-cystine in exchange for intracellular glutamate or [^{3}H]-glutamate in exchange for unlabeled intracellular glutamate (Bannai,1986). The latter approach is routinely preferred because of the unstable nature of ^{35}S radiolabel (Shih and Murphy,2001) and because it identifies specifically the transport function (i.e., substrate specificity) of system xc- (Bridges et al., 2001). In the present experiment, Na^+-independent [^{3}H]-glutamate uptake was examined in ventral striatum slices as previously described (Melendez et al.,2005). Briefly, mice were decapitated, and the ventral striatum was rapidly dissected and cut into 350 × 350-μm prism-shaped slices with use of a McIllwan tissue chopper (Vibratome, St. Louis, MO). The slices were washed for 30 min at 37°C in oxygenated sodium free Krebs-Ringer's solution phosphate buffer (140 mM choline chloride, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5 mM HEPES, 10 mM glucose, and 1 mM $MgCl_2$) with a final pH of 7.4. Glutamate uptake measurements were initiated by adding L-[^{3}H]-glutamate (250 nM, 51 Ci/mmol; Perkin Elmer, Boston, MA) in the presence of 1 to 10 μM unlabeled L-glutamate in a final volume of 250 μl of oxygenated buffer. This range of glutamate concentration was chosen because it encompasses extracellular glutamate levels in rat brain (Baker et al.,2003). After incubation at 37°C for 15 min, uptake was terminated by washing the slices in ice-cold nonradioactive choline-containing buffer. Slices were then solubilized with use of 1% sodium dodecyl sulfate, and the level of radioactivity was determined using a liquid scintillation counter, and protein content in the slices was measured using the Bradford assay.

4.5. xCT antibody production and Western blotting

Rabbit xCT polyclonal antibody was generated using a synthetic peptide corresponding to 15 amino acid sequence of N-terminal mouse xCT protein (VATISKGGYLQGNMS) with 8 branch multiple antigen peptide. More detailed evaluation of this antibody has been published elsewhere (Szumlinski et al.,2004). Brain tissue from drug-naïve Nrf2 KO and WT littermates was dissected, immediately frozen and kept in a − 80 °C freezer until analysis. Tissue was prepared and Western blotting conducted as described in detail elsewhere (Toda et al.,2003). Briefly, the frozen tissue was manually homogenized in RIPA buffer containing protease inhibitor cocktail (Roche). Protein concentration was measured using the Bradford assay (Pierce). Samples (20 μg per lane) were run in 10% Tris-acrylimide gels, separate gels per brain area. Incubation with the xCT antibody (1:200) was followed by incubation in anti-rabbit horseradish peroxidase conjugated antibody (1:20,000; Amersham), and reactivity was detected using ECL chemiluminescence substrate (Amersham). Densitometric analysis of Western blots was performed with the Image J program (version 1.36b). Band intensities were measured by taking the pixels integrated density.

4.6. Statistical Analysis

All data were statistically evaluated using analyses of variance (ANOVA). When appropriate, the data were decomposed for simple effects, and post hoc comparisons were made using a Fisher's least significant difference test.

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Abbreviations

ARE, antioxidant responsive element

DA, dopamine: DOPAC, dihydroxyphenylacetic acid
HVA, homovanillic acid
Keap1, Kelch-like ECH associating protein 1
METH, methamphetamine
Nrf2, NF-2 related factor 2
Nrf2 KO, mouse with an homozygous deletion of Nrf2 gene
Nrf2 WT, wild-type littermates of Nrf2 KO mice
PFC, prefrontal cortex
ROS, reactive oxygen species
system xc-, cystine/glutamate exchanger
xCT, catalytic chain of system xc-

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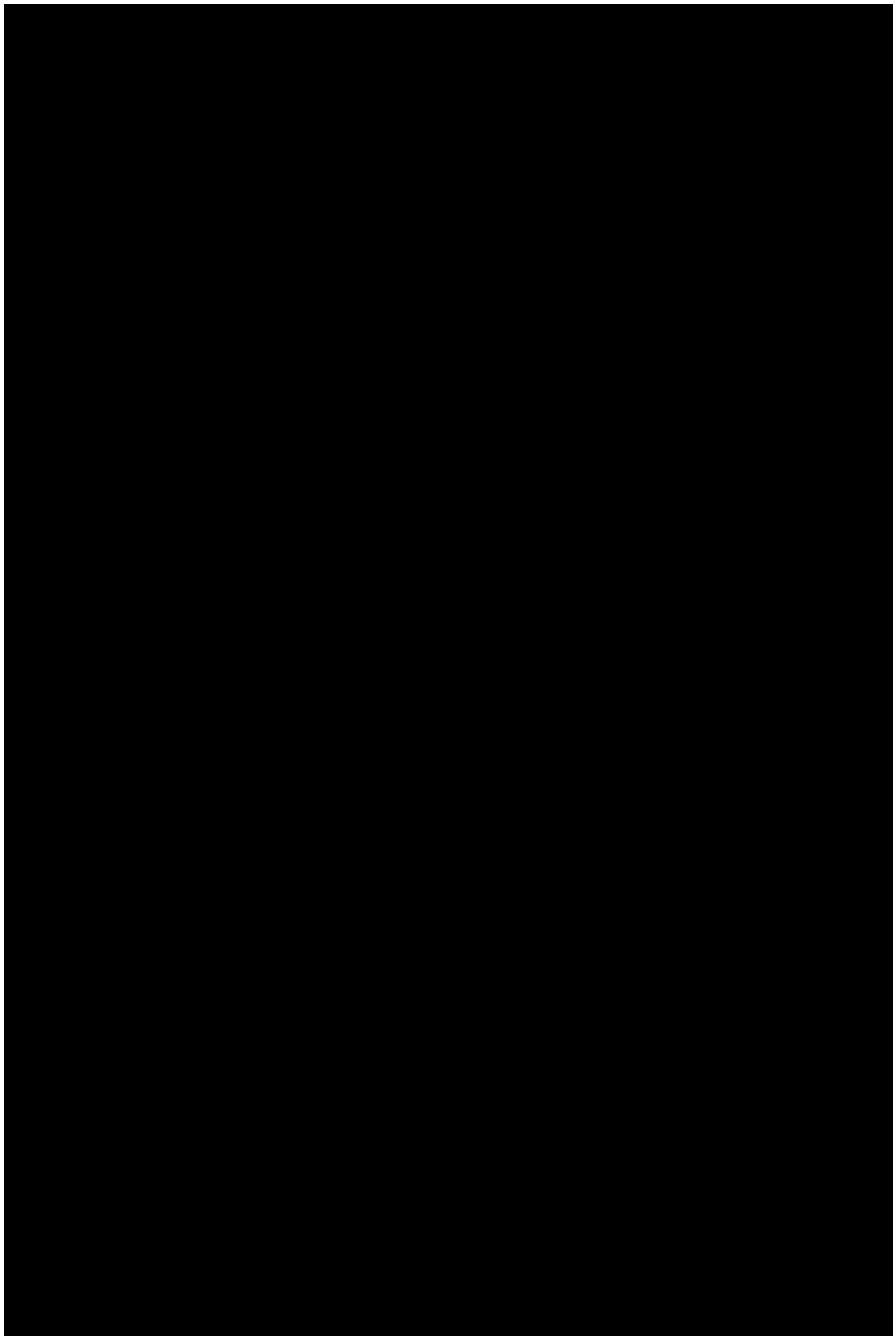

Figure 1.

Basal system xc- activity and the expression of xCT protein are not altered by Nrf2 gene deletion. **a)** Na^+-independent L-[^{3}H]-glutamate uptake was used to estimate activity of cystine/glutamate exchanger. Data are expressed as mean ± sem at three different L-glutamate concentrations in the presence of 0.25μM of L-[H^3]-glutamate. (N= 5 in each group). **b)** Representative Western blot for xCT and calnexin protein in PFC, ventral and dorsal striatal homogenates from littermate Nrf2 WT and KO mice. **c)** Densitometric measurement of relative band intensity (in arbitrary units) for xCT. N= 4-6 for each genotype, statistically analyzed separately in each brain area. All data are shown as mean ± sem. $p < 0.0001$, * compared to 1nM and ** compared to 5 nM.

Figure 2.

Four cocaine (10 mg/kg i.p.) conditioning sessions results in equivalent place conditioning and locomotor sensitization in Nrf2 KO and WT mice. **a and b)** Time spent in the drug paired-side during the Pre- and Post-Test trials in the place conditioning apparatus (panel a), and total beam breaks during each 15 min conditioning session (panel b). Data are shown as mean ± sem, and were analyzed using a 3-way ANOVA with repeated measures over trial, and between subjects factors being drug and genotype. WT N= 26 and KO N=22, between Saline and Cocaine groups. **c)** Total distance traveled (cm) over 60 min after injecting saline or cocaine. Data are show as mean ± sem and were evaluated using a two-way ANOVA with repeated measures over drug. N= 5 for WT; N= 7 for KO.

* $p < 0.05$, compared to the Pre-test (panel a), or first injection (panel b), or saline (panel c)

Figure 3.

Equivalent behavioral effect of METH on Nrf2 KO and WT. Four METH injections were administered at 2 hrs intervals. **a)** Total distance traveled over the first 20 min after the first and last METH injection. Data are shown as mean ± sem and were analyzed using a 3-way ANOVA. N= 7-9 in each group. METH 5= METH 5 mg/kg, i.p.; METH 10= METH 10 mg/kg, i.p. **b)** Binge METH administration did not produce differences between Nrf2 KO and WT in xc- activity in ventral striatal slices. Data are expressed as picomol per mg of protein per minute at three different concentrations of L[H^3]-glutamate. (N=3 per group). * $p< 0.05$, comparing METH to saline ; # $p< 0.05$ comparing the 1st and 4th injection (panel a). * $p< 0.005$ compared to 5 μM; ** $p< 0.0001$ compared to 150 μM (panel b).

Table 1

Two weeks after repeated METH dopamine and its metabolites were measured in the dorsal and ventral striatum.

			DA	DOPAC	HVA
Ventral Striatum	**Saline**	WT	582.1 ± 92.7	181.5 ± 23.1	94.3 ± 13.1
		KO	547.8 ± 70.7	150.9 ± 26.4	87.0 ± 12.3
	Meth 5	WT	231.2 ±42.8 *	163.9 ± 29.6	71.9 ± 10.9
		KO	333.8 ± 66.6 *	204.1 ± 51.5	94.6 ± 19.3
	Meth 10	WT	313.9 ±79.6 *	94.2 ± 10.5 * δ	63.0 ± 9.95
		KO	364.5 ± 74.3 *	107.1 ± 12.9 * δ	68.1 ± 10.3
Dorsal Striatum	**Saline**	WT	889.9 ± 86.1	131.25 ± 14.4	99.6 ± 11.6
		KO	969.2 ± 158.9	114.8 ±24.1	99.7 ± 13.2
	Meth 5	WT	429.7 ± 95.1 *	98.1 ± 23.4	92.1 ± 11.7
		KO	341.6 ± 51.6 *	80.7 ±11.6	109.8 ± 14.9
	Meth 10	WT	152.5 ± 44.9 * δ	47.1 ± 14.1 * δ	48.1 ± 8.3 * δ
		KO	177.2 ± 46.3 * δ	42.8 ± 8.3 * δ	49.1 ± 9.6 * δ

Both doses of METH binge induced a significant DA depletion in dorsal and ventral striatum, regardless the genotype. Similar effects were also seen with DA metabolites, but only at the highest dose of METH altered DOPAC and HVA in the dorsal striatum and DOPAC in the ventral striatum. Data are shown as mean ± sem and expressed as picomoles per mg of protein. n = 7-9 in each group.

* p< 0.05, comparing METH with saline ;

δ p< 0.05, comparing METH 5 with METH 10