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NrCAM regulating neural systems and addiction related behaviors

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Abstract

We have previously shown that a haplotype associated with decreased NrCAM expression in brain is protective against addiction vulnerability for polysubstance abuse in humans and that *Nrcam* knockout mice do not develop conditioned place preferences for morphine, cocaine, or amphetamine. In order to gain insight into NrCAM involvement in addiction vulnerability, which may involve specific neural circuits underlying behavioral characteristics relevant to addiction, we evaluated several behavioral phenotypes in *Nrcam* knockout mice. Consistent with a potential general reduction in motivational function, *Nrcam* knockout mice demonstrated less curiosity for novel objects and for an unfamiliar conspecific, showed also less anxiety in the zero maze. *Nrcam* heterozygote knockout mice reduced alcohol preference and buried fewer marbles in home cage. These observations provide further support for a role of NrCAM in substance abuse including alcoholism vulnerability, possibly through its effects on behavioral traits that may affect addiction vulnerability, including novelty seeking, obsessive compulsion and responses to aversive or anxiety-provoking stimuli. Additionally, in order to prove glutamate homeostasis hypothesis of addiction, we analyzed glutamatergic molecules regulated by *NRCAM*. Glutaminase appears to be

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involved in NrCAM-related molecular pathway in two different tissues from human and mouse. An inhibitor of the enzyme, PLG, treatment produced, at least, some of the phenotypes of mice shown in alcohol preference and in anxiety-like behavior. Thus, NrCAM could affect addiction-related behaviors via at least partial modulation of some glutamatergic pathways and neural function in brain.

Keywords

behavior; cell adhesion molecule; glutamate

Introduction

We have previously shown that a neuronal cell adhesion molecule NrCAM is associated with drug abuse vulnerability (Ishiguro et al., 2006). The role of NrCAM in polysubstance abuse vulnerability was initially proposed based on the results of genome wide association study (GWAS) in humans that examined sets of abusers who met criteria for abuse of and dependence on multiple classes of drugs. Further study using an animal model supported this initial observation in humans, demonstrating that gene knockout of *Nrcam* reduced the rewarding effects of morphine, cocaine, and amphetamine in mice (Ishiguro et al., 2006). Since each of these substances produces rewarding effects through distinct molecular targets in the brain as demonstrated by gene knockout studies (Sora et al., 2001a; Sora et al., 2001b; Sora et al., 1997; Sora et al., 1998; Takahashi et al., 1997), NrCAM is likely to affect a common pathway or pathways underlying drug abuse vulnerability.

Another interesting possibility, though not mutually exclusive with the first possibility, is that NrCAM may be responsible for certain characteristic behavioral traits, to which variations in the *NRCAM* gene could contribute, that predispose individuals to addiction and maybe any other related phenotypes. Interestingly, associations between *NRCAM* and other psychiatric disorders, schizophrenia and autism, have also been reported (Kim et al., 2009; Marui et al., 2009).

Previous studies have repeatedly reported a high correlation between novelty seeking assessed by the Temperament and Character Inventory (TCI) or Tridimensional Personality Questionnaire (TPQ) test and substance abuse (Evren et al., 2007; Lukasiewicz et al., 2008; Martinotti et al., 2008; Sher et al., 2000). Additionally, subgroups of addicted patients show differences in anxiety and/or harm avoidance behavior (Ducci et al., 2007; Finn et al., 2002; Hosak et al., 2004). Obsessive compulsive behavior was found in substance use disorders (Friedman et al., 2000; Hollander et al., 2007; Mancebo et al., 2009). Although our previous study demonstrated involvement of NrCAM in reward for abused drugs in the conditioned place preference paradigm, it remains unclear if NrCAM is involved in the development of preference. Therefore in the present study, we examined *Nrcam* knockout mice for behaviors that may model some of the behavioral characteristics or comorbid conditions relevant to human abusers' predisposition to addiction. Mice with a lack of *Nrcam* demonstrate alterations in brain function that may reflect the changes of the neuro-behavioral phenotypes in addiction. Previous study had presented that *Nrcam* knockout mice

exhibit selective changes in anxiety-like behavior and reaction to stress (Matzel et al., 2008). However, the analysis by Moy et al. (Moy et al., 2009) did not support the role of *Nrcam* in anxiety-like behavior measured by elevated-plus maze, while male *Nrcam*-null mice had significantly decreased sociability (Moy et al., 2009). To resolve the controversy and certain phenotypes related to addiction, further analyses are needed by additional animals and behavioral tests in this study.

On the other hand, glutamatergic (Grueter et al.; Kalivas et al., 2009; Knackstedt et al.), as well as serotonergic (Kirby et al.) functions in brain may contribute to addiction vulnerability and other related phenotypes. NrCAM has important roles in normal brain development, including the development of the hippocampus (Custer et al., 2003; Lustig et al., 2001; Lustig et al., 1999; Petralia et al., 2005; Sakurai et al., 2001; Sakurai et al., 1997), and might therefore have a role in adult brain plasticity as well. Indeed, one possibility for the nature of NrCAM involvement in addiction vulnerability is that NrCAM may play a role in neuronal plasticity, affecting addictive behavior through modulation of plasticity in reward related circuits that underlies responses to conditioned stimuli as in the conditioned place preference paradigm, as well as other phenotypes shown in the study. We therefore attempt to evaluate glutamatergic molecules regulated in low NrCAM expression tissues in this study.

Materials and Methods

Subjects

Two strains of *Nrcam* knockout mice were used in this study. As first group, *Nrcam* knockout mice were generated by Sakurai et al (Sakurai et al., 2001), and the heterozygous Brother Sister mating pairs have been obtained from the same litter and were used as breeding pairs to produce cohorts used in this study. As second group, two *Nrcam* male mice of same litter were mated with C57BL/6JmsSLC female mice (Japan SLC, Inc, Shizuoka, Japan) to generate heterozygote mice with B6 background and the *Nrcam* mouse strain which was originally generated by Sakurai et al (Sakurai et al., 2001), in order to increase sensitivity to alcohol, and to highlight the marble-burying behavior as B6 mice buried certain amount of the marbles as shown in the previous study (Deacon and Rawlins, 2005; Nicolas et al., 2006). We used the F1 generation (*Nrcam* wild and heterozygote knockout type) for alcohol preference test and for marble-burying behavior.

The studies were approved by either the Committee of Animal Study at University of Tsukuba or the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse. (See details in Supplementary Materials and Methods).

Morphine Analgesia

Male mice (N = 6–11 per genotype) were tested for nociceptive and anti-nociceptive effects of morphine, using the hot-plate (supraspinal response) and the tail-flick (spinal response) tests. After establishment of initial baseline nociceptive thresholds with four repeated assessments, morphine effects by injection s.c. at 20 min intervals with an ascending

cumulative dosing regimen (Hall et al., 2003; Sora et al., 1997). (See details in Supplementary Materials and Methods).

Alcohol Preference

Male F1 generation *Nrcam* knockout mice with B6 heterozygote background (13 wild and heterozygote *Nrcam* knockout mice each) were tested. Each mouse was housed separately in home cage where mice could freely access two nozzles for regular water or alcohol containing water. The consumption of the alcohol by drinking 16% alcohol during a week was measured. (See details in Supplementary Materials and Methods).

Novel object preference

We used two protocols (See details in Supplementary Materials and Methods): protocol #1 was a slightly modified protocol previously described (Ballaz et al., 2007; Bienkowski et al., 2001; Ennaceur and Delacour, 1988), and protocol #2 was a novel variant of protocol #1 to produce less psychological pressure on mice that might feel anxious to directly contact to the figures. Genotype and sex effects were analyzed in the test (N = 2–39 for protocol #1, and N = 16–45 for protocol #2 for each genotype). The time spent exploring each of the objects in terms of physical contact or rearing towards the object was measured.

Sociability

Genotype and sex effects were analyzed for preference for a social object (N = 14–39 for each genotype). Experimental protocols and the test cage were slightly modified from a protocol described in the literature (Moy et al., 2004), to measure preference for a social object. A target mouse was placed inside of the wire cage for a test mouse to allow nose contact, in the experimental compartment. (See details in Supplementary Materials and Methods).

Anxiety

Three methods were performed to evaluate anxiety. Details are described in Supplementary Materials and Methods. Genotype and sex effects on anxiety (N = 12–21 for each genotype) in Zero Maze using the Video Tracking System (Med Associates, Inc., St. Albans, VT, USA) were analyzed in 5 min, and percent time spent in the open sections was measured.

Marble-burying behavior

The marble-burying behavior of male F1 generation *Nrcam* knockout mice with B6 heterozygote background (9 wild and heterozygote genotype each) was recorded using modified protocol as described (Umathe et al., 2009). The total number of marbles buried in 40 min was compared between wild and heterozygote mice. (See details in Supplementary Materials and Methods).

Transcriptome analysis in NrCAM knockdown tissue

Double strand siRNAs were prepared by Custom siRNA Synthesis (Takara Bio Inc, Otsu, Shiga) that have *NRCAM* specific sequence. The siRNA was administered to T98G cells with TrasIT-TKO Transfection Reagent (Mirus, Madison, WI). Total RNA was extracted

from the cells and cDNA was synthesized. Microarrays were used to screen for differential gene expression between cells treated with siRNA of *NRCAM* sequence and random sequence. (See details in Supplementary Materials and Methods).

We focus on a gene that was potentially regulated more than 50% accompanied with lower expression of *NRCAM* and encodes glutamate or serotonin neuron-related molecule, which was glutaminase gene (*GLS*) in the study. Mouse *Gls* gene expression in prefrontal cortex was also compared between *Nrcam* knockout heterozygote mice and homozygote mice (6 +/+ mice vs. 6 +/- mice), using TaqMan gene expression assays. The difference of *Gls* gene expression between *Nrcam* knockout heterozygote mice and homozygote mice was confirmed in ventral midbrain (5 +/+ mice vs. 7 +/- mice). (See details in Supplementary Materials and Methods).

Effect of glutaminase inhibitor in mice behavior

GLS was inhibited by prolyl-leucyl-glycinamide (PLG) (Koyuncuoglu et al., 1992). In their previous study, PLG was injected to mice from peripheral (s.c.), to examine its effect on morphine-induced tolerance and dependence. Therefore, we also did peripheral administration of PLG (not s.c. but i.p.) in this study. An effect of PLG was evaluated in C57BL/6JmsSLC mice for similar phenotypes in *Nrcam* knockout mice, in order to evaluate the specific phenotypes influenced by modification of brain *Nrcam*-Gls related neural pathway. The behavioral tests were conditioned place preference (CPP), alcohol preference, novel object seeking, sociability, Zero maze, and marble burying tests. (See details in Supplementary Materials and Methods).

Briefly, PLG (25 mg/kg, i.p.) was administered i.p. 30 min prior to drug treatment in each experiment. CPP in male mice (Japan SLC, Inc, Shizuoka, Japan) (age, 8 wk; weight, 20–25 g; N = 8–16 for each group) were examined by using same protocol that we previously reported (Ishiguro et al., 2006). Locomotion in different groups of male mice (Japan SLC, Inc, Shizuoka, Japan) (age, 8 wk; weight, 20–25 g; N = 5–8 for each group) treated with morphine (20 mg/kg i.p.), cocaine (5 mg/kg s.c.), methamphetamine (2 mg/kg, i.p.) or saline were monitored for locomotor activity in their home cages. Locomotor activity of mice treated with each drug after PLG pretreatment was compared with that of mice treated with the drugs after saline pretreatment. Alcohol preference was assessed in a same protocol described above except using PLG (25 mg/kg) or saline injection daily to C57BL/6J male mice (n = 10 each, Charles River Japan, Yokohama, Japan).

Statistical Analysis

The analytical methods were described in Supplementary Materials and Methods.

Results

Morphine analgesia

There was no significant effect of genotype for baseline analgesia either in the hot-plate tests ($F[2,27] = 0.5$, ns) or tail-flick ($F[2,28] = 2.4$, ns). Baseline latencies for the final habituation trial in the hot-plate test were *Nrcam* +/+ 7.4 ± 5.4 , *Nrcam* +/- 6.2 ± 3.6 , *Nrcam* -/- $8.8 \pm$

4.6. Baseline latencies for the final habituation trial in the tail-flick test were *Nrcam* *+/+* 3.9 ± 0.7 , *Nrcam* *+/-* 3.9 ± 0.5 , *Nrcam* *-/-* 3.4 ± 0.2 . Morphine produced significant analgesic effects in both the hot-plate (Figure 1A; $F[2,27] = 54.1$, $p < 0.0001$) and tail-flick tests (Figure 1B; $F[2,28] = 64.4$, $p < 0.0001$). In each case analgesic effects reached the maximum at a dose of 20 mg/kg morphine and were nearly at maximum at 10 mg/kg. There were no significant effects of genotype in either the hot-plate ($F[2,27] = 0.4$, ns) or tail-flick ($F[2,28] = 2.1$, ns) tests, nor were there any significant interactions between genotype and morphine dose for either the hot-plate ($F[2,27] = 0.5$, ns) or tail-flick ($F[2,28] = 0.2$, ns) tests. There was a slight trend toward reduced sensitivity to morphine at 3 mg/kg in *Nrcam* *-/-* mice (Figure 1B), but this trend was not statistically significant.

Alcohol preference

Heterozygote *Nrcam* knockout mice developed alcohol preference by drinking high concentration ethanol. Total alcohol consumption by drinking 16% in 7 days were 14.6 ± 1.8 mg/kg in *+/+* mice and 9.8 ± 1.0 mg/kg in *+/-* mice, which was significantly different between the genotypes ($F[1,25] = 5.7$, $P = 0.025$, Figure 2).

Novel object preference

Using protocol #1, where mice could directly contact objects on the floor, the difference between time spent investigating the novel object and one spent at same area in the familiarization session (novel-habituation) was significantly different between genotypes ($F[2,2] = 9.1$, $P = 0.0003$, Figure 3A), while weak effect was found in sex x genotype ($F[2,2] = 4.5$, $P = 0.01$), but no clear effect found from sex ($F[1,1] = 0.6$, ns). Post-hoc analysis showed that *Nrcam* *+/+* mice and *+/-* mice spent more time investigating the novel object whereas *-/-* mice showed reduced preference for the novel object ($P = 0.003$, $P = 0.04$, respectively). Using protocol #2, where approach to the objects was measured in terms of rearing, the difference in rearing counts towards the novel and familiar objects showed similar effect from genotype ($F[2,2] = 3.4$, $P = 0.03$, Figure 3B) but not from sex ($F[1,1] = 0.6$, ns) nor genotype x sex ($F[2,2] = 1.3$, ns). Post-hoc analysis showed that *Nrcam* *-/-* mice evinced less rearing towards the novel object (post hoc comparison $P = 0.02$, versus *+/+* mice), while there was similar trend of difference between *+/-* and *+/+* mice ($P = 0.06$).

Sociability

There was a significant difference found in the behavior between genotypes ($F[2,2] = 3.4$, $P = 0.04$, Figure 4). There was no effect of sex ($F[1,1] = 0.1$, ns) nor sex x genotype ($F[2,2] = 1.5$, ns). *Nrcam* *+/+* mice exhibited a greater preference for the chamber containing the unfamiliar mouse than the empty chamber, while knockout mice tended to avoid the novel target mouse. *Nrcam* *-/-* and *+/-* mice spent substantially less time investigating the unfamiliar mouse ($P = 0.05$), while *+/-* mice were intermediate in their preference. The number of entries into each test chamber were not significantly different between genotypes (data not shown).

Anxiety

There was significant difference in the percent time spent in the open section of the zero maze among the *Nrcam* genotypes ($F[2,47] = 3.4$, $P = 0.01$, Figure 5), while there was no effect from sex ($F[1,1] = 0.02$, ns) and sex x genotype ($F[2,2] = 1.1$, ns). Post-hoc analysis showed that *Nrcam* $-/-$ mice spent significantly more time in the open area compared with $+/+$ mice ($P < 0.01$).

Marble-burying behavior

Marble burying behavior is genetically regulated. Number of marbles buried by *Nrcam* $+/+$ mice (3.7 ± 0.6) was significantly more than that by *Nrcam* $+/-$ mice (2.0 ± 0.4) ($F[1,17] = 5.3$, $P = 0.04$, Figure 6).

Transcriptome analysis in NrCAM knockdown tissue

Microarray analysis revealed one of glutamatergic genes, *GLS* gene, was down-regulated more than 50% accompanied with lower expression of *NRCAM* (66 % down-regulated) in cells treated with siRNA. *GLS* gene was a sole neuron-related molecule among 15 candidate genes (listed in Supplemental Information), and its down-regulation in the cultured cells was confirmed by TaqMan analysis. Mice *GLS* gene expression were also downregulated in *Nrcam* heterozygote knockout mice in comparison to those in wild type mice ($F[1,10] = 4.9$). The difference of *GLS* expression was confirmed in ventral midbrain ($P = 0.05$).

CPP and locomotion in drug responses altered by PLG

Mice treated with PLG (25 mg/kg, i.p.) display reduced preferences for the places where they received morphine (20 mg/kg, i.p.), cocaine (5 mg/kg, s.c.) or methamphetamine (2 mg/kg, i.p.) in comparison to saline treated control mice, observed in CPP test ($F[1,12] = 6.4$, $P = 0.03$, $F[1,26] = 4.6$, $P = 0.04$, $F[1,25] = 10.6$, $P = 0.003$, Figure 7A). Mice pretreated with PLG (25 mg/kg, i.p.) reduced preference to cocaine, methamphetamine and morphine. All of the abused drugs used increased locomotion of mice, pretreatment with PLG (25 mg/kg, i.p.) inhibited the drug-induced hyperlocomotion, respectively. PLG alone did not change locomotion significantly during 3 h after the injection, compared with that in mice with saline treatment (Figure 7B).

Alcohol preference, novel object seeking, sociability, anxiety, and marble burying behavior altered by PLG

Although mice treated with PLG (25 mg/kg, i.p.) seemed to reduce preferences to the novel object, PLG did not induce similar alterations in phenotypes of *Nrcam* knockout mice with regard to novelty seeking, sociability or marble burying behavior ($F[1,19] = 0.96$, $P = 0.34$, Figure 8A, $F[1,19] = 0.05$, $P = 0.83$, Figure 8B, and $F[1,25] = 0.03$, $P = 0.86$, Figure 8C). In contrast, mice pretreated with PLG (25 mg/kg, i.p.) reduced anxiety-like behavior observed in Zero maze ($F[1,18] = 11.6$, $P = 0.003$, Figure 9), which is similar to that observed in the phenotype of *Nrcam* knockout mice. Those mice tended to develop less alcohol preference when they had access to drink high concentration ethanol. Total alcohol consumption by drinking 16% in 7 days were 11.4 ± 1.2 mg/kg in $+/+$ mice and 8.6 ± 0.8

mg/kg in +/- mice, which appeared to be a trend of difference between the genotypes ($F[1,18] = 3.6$, $P = 0.07$, Figure 10).

Discussion

The present study documented that 1) *Nrcam* knockout mice did not show apparent difference in nociceptive responses and morphine analgesia, 2) *Nrcam* knockout mice showed less preference to novel object, 3) *Nrcam* knockout mice showed less sociability to unfamiliar mouse, and 4) *Nrcam* knockout mice showed less anxiety in zero maze. These data can therefore be interpreted that in these mice there is reduced novelty seeking (from above findings 2 and 3) and also less anxiety-like behavior or harm avoidance (from above finding 4). Additionally, while we first examined sex effect on the phenotypes, we did not see significance in novelty object seeking, sociability and Zero maze tests.

In comparisons with previous studies from other researchers, studies from Matzel (Matzel et al., 2008) and this study seems to be in agreement for a role of *Nrcam* in anxiety-like behavior, although it had not been observed in elevated plus maze by Moy et al. (Moy et al., 2009). A lack of sociability has been observed in male mutant mice in Moy's study, while it was not observed in female mutant mice in the study. Since there was no sex effect on the sociability in this study, we concluded that *Nrcam* could affect sociability, even if the effect size might be weak.

In addition to these findings, we evaluated glutaminase that appears to be involved in *NrCAM*-related molecular pathway in two different tissues from human and mouse. Since a reduction of *NrCAM* induces down-regulation of glutaminase, an inhibitor of the enzyme could explain at least some part of addiction-phenotypes observed in *Nrcam* knockout mice. Indeed, PLG treatment produced similar phenotypes of mice in alcohol preference and in anxiety-like behavior.

In our previous study, *Nrcam* knockout mice demonstrated reduced rewarding effects with several types of addictive substances (Ishiguro et al., 2006). These findings support our human genetics findings that reduced *NRCAM* gene expression by particular *cis*-acting haplotypes was protective against substance abuse vulnerability (Ishiguro et al., 2006). The knockout mouse study may be especially relevant to allelic variations in humans that produce approximately 50% differences in *NRCAM* expression, since heterozygous also show phenotype similar to homozygous knockout mice. It is noteworthy that both animal and human genetic studies showed an apparent role of *NrCAM* in addiction vulnerability for several different classes of abused drugs that act through very distinct molecular mechanisms. *NrCAM* could therefore play an important role in a common pathway underlying the rewarding or addictive behavior; this role could be in basic motivational processes that underlie addiction, in the maintenance of neuronal connections important for the reinforcing or conditioned effects produced by drugs of abuse, or in some other mechanisms perhaps in an underlying phenotype relevant to drug addiction (e.g., personality traits such as novelty seeking or harm avoidance).

In addition to rewarding effects, morphine causes analgesia, an effect that can lead to its abuse. As the reward and analgesia effects could partially, at least, share the same molecular pathways in the brain. Our experiments however, did not show any effect of *Nrcam* in the Hot-plate test or the Tail-flick test. Therefore, we concluded that the major molecular pathways that are regulated by NrCAM must be other than the ones involved in analgesia produced by morphine.

Thus, there are still a number of possibilities for what role *NRCAM* may play in addiction. In the next series of experiments, we explored the possibility that NrCAM plays roles in establishing behavioral characteristic traits that predispose individuals to drug addiction. Cloninger described four primary psychobiological dimensions of temperament: novelty seeking, harm avoidance, reward dependence, and persistence. These factors consist of innate biases in perceptual memory and habit formation (Cloninger et al., 1993), which are of obviously potential importance for understanding behavioral traits associated with addiction, but more importantly, they may allow the parsing of addicted patients into distinct groups related to particular traits that may predispose individuals to addiction.

Among those traits, novelty seeking is the most strongly associated with the use of multiple addictive substances (Conway et al., 2003). In addition, novelty seeking traits tend to be found in individuals addicted to wide variety of classes of addictive substances, including nicotine (Wills et al., 1994), ethanol (Finn et al., 2002), marijuana (Lynskey et al., 1998) and other addictive drugs (Wills et al., 1994). Previously, we found that *Nrcam* knockout mice showed reduced conditioned place preference for several types of addictive substances (Ishiguro et al., 2006), which is in accord with a role of *NRCAM* in addiction through its effects on novelty seeking. In support of this hypothesis, we found that *Nrcam* knockout mice showed less preference for both novel objects and novel social stimuli (Figure 2 and 3), for which sex difference seemed not to have significant effects in the animal models used in this study.

NRCAM might also have effects on other traits that have been hypothesized to play a role in addiction, such as harm avoidance. However, previous analyses of the harm avoidance traits in abusers have been much less consistent than findings for novelty seeking; For example, low harm avoidance scores have been found in early-onset alcoholics (Finn et al., 2002), while high scores in methamphetamine abusers (Hosak et al., 2004) and late-onset alcoholics (Ducci et al., 2007). And no differences have been found for some addictive drugs such as cocaine (Gerra et al., 2000) or opiates (Gerra et al., 2008). Although translating harm-avoidance as a trait to simple animal models may be debatable, the present study did find reduced anxiety in the Zero Maze in *Nrcam* knockout mice compared with wild-type controls (Figure 4A and 4B). Collectively, these results indicate that NrCAM may play some role in establishing harm avoidance traits or anxiety. However, since differences in harm avoidance in humans appears to have different effects in addictions to distinct classes of addictive drugs, it would appear that the role of *NRCAM* in harm avoidance may not have a significant role in a common pathway underlying addiction vulnerability to multiple classes of addictive drugs.

Marble-burying behavior in rodents is an unconditioned defensive reaction, which is not associated with physical danger, and does not habituate upon repeated testing, and in addition was altered by anxiolytics (Njung'e and Handley, 1991). Restraint stress significantly decreased the marble-burying behavior in mice, while social isolation stress increased it (Umathe et al., 2009). On other hand, marble burying is not correlated with other anxiety-like traits, not stimulated by novelty, and is a repetitive behavior that persists/perseveres with little change across multiple exposures (Thomas et al., 2009). Total number of marbles buried was also considered as an index of obsessive-compulsive behavior indicating aberrant ethological responses to attractive stimuli (Umathe et al., 2009) and an animal model to screen anti-OCD drugs (Joel, 2006). Several publications discussed that OCD could exist in substance use disorders, although biological mechanism in the comorbidity has not been identified (Friedman et al., 2000; Hollander et al., 2007; Mancebo et al., 2009). Interestingly, marble burying behavior was associated with ethanol withdrawal in mice (Umathe et al., 2008). Bruins et al. (2008) examined effects of several antipsychotics on marble burying behavior which suggested involvement of dopaminergic and serotonergic systems in the behavior (Bruins Slot et al., 2008). In male mice it is markedly attenuated by acute administration of SSRI or tricyclic antidepressants (Ichimaru et al., 1995). MDMA, primarily by increasing 5-HT function, but not by increasing dopamine function, acts like several anxiolytic drugs in this behavior (Saadat et al., 2006). Indeed, GLS inhibitor did not alter the behavior in this study.

Notably glutamate neural function has pivotal roles in addiction, including reward, reinforcement, cognitive process for learning and memory (Gass and Olive, 2008; Kalivas et al., 2009; Tzschentke and Schmidt, 2003; Uys and LaLumiere, 2008), and marble burying behavior is also reduced by glutamate receptor antagonists via NMDA but not via AMPA receptor as well (Egashira et al., 2008). While ligands for metabotropic glutamate receptors are well documented to be potential therapeutic targets for addiction (Olive, 2009), glutaminase had not been investigated in almost two decades since it was investigated for physical tolerance/dependence by morphine (Koyuncuoglu et al., 1992). In this study, PLG inhibited reward and response to hyperlocomotion induced by morphine, cocaine and methamphetamine demonstrated in the CPP test. In addition, PLG inhibits development of alcohol preference and reduces anxiety in mice. However, in contrast, glutaminase inhibitor PLG injection i.p. did not show a difference in some addiction-Nrcam-related behavior, which are novelty-seeking, sociability and marble burying. Thus, those addiction-related behaviors are not characterized by glutamate function. As mentioned above, NrCAM may be involved in addiction, via non-glutamatergic systems, such as serotonergic or dopaminergic system, too. However, we noticed the limitation of the study: While an effect of PLG was evaluated in C57BL/6JJmsSLC, the genetic background of *Nrcam* knockout mouse is Swiss Webster. Although we found the similar effect on alcohol preference in both of C57BL/6JJmsSLC mice and heterozygote C57BL/6JJmsSLC background *Nrcam* knockout mice, the deference of some behavioral/phenotypic responses to PLG might be caused by the genetic background.

In summary, NrCAM seems to affect the development of the traits or behavior that are associated with responses to novel stimuli and appetitive motivational states, which have

been strongly implicated in addiction and related to glutamate function and certainly other neural circuits including mesolimbic dopamine function. NrCAM also seems to modulate processes related to responses to aversive stimuli and perhaps anxious traits that have been sometimes described as harm avoidance. However, the latter findings may not necessarily fit well with the apparently broad effects of NrCAM on multiple classes of addictive drugs. By evaluating specific roles of NrCAM in circuitry responsible for motivational processes, particularly those which may affect responses to novel stimuli or stimuli that are predictive of reinforcers, and those which may underlie the development of impulsive and compulsive responses to drug rewards, novel pharmacological targets may be identified to develop treatments for addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Morphine analgesia in *Nrcam* knockout mice

1A: Hot-plate, 1B: Tail-flick. There was no effect of NrCAM genotype. Vertical axis shows response time ratio against cut-off time as percentage analgesia. Horizontal axis shows morphine dose. Sample size shown in parenthesis. * $P < 0.05$ Data are expressed as mean $\pm$ the standard error of the mean.

Figure 2.

Alcohol Preference in *Nrcam* knockout mice

Mice are F1 generation littermates born from C57BL/6JmsSLC by mating with 129 background *Nrcam* knockout mouse. *Nrcam* homozygote mice and heterozygote mice were compared for consumption of ethanol. Data are expressed as mean $\pm$ the standard error of the mean of alcohol consumption from 16% alcohol by drinking in seven days.

Figure 3.

Novel object preference in *Nrcam* knockout mice

3A: Difference in time spent near the novel object compared to the familiar object. 3B:

Difference of time of rearing towards the novel and familiar objects. Sample size shown in parenthesis for ++: wild type, +/-: heterozygote type and -: mutant type. Data are expressed as mean $\pm$ the standard error of the mean.

Figure 4.

Social preference in *Nrcam* knockout mice

Difference of time spent in the compartment containing the unfamiliar target mouse. Sample size shown in parenthesis for ++: wild type, +/-: heterozygote type and -: mutant type. Data are expressed as mean $\pm$ the standard error of the mean.

Figure 5.

Anxiety traits in *Nrcam* knockout mice

5: Anxiety in the Zero Maze. Vertical axis shows percentage time spent in the open section of the zero maze. Sample size shown in parenthesis for ++: wild type, +-: heterozygote type and -: mutant type.

Figure 6.
Marble Burying Test in *Nrcam* knockout mice
Data are expressed as mean $\pm$ the standard error of the mean for the number of burying marbles.

Figure 7.

PLG and drug response in CPP and locomotion

C57BL/6JmsSLC mice were treated with saline, morphine, methamphetamine, or cocaine. 7A: CPP was measured by differences in time spent on the side paired with either drug (post exposure – pre exposure) are displayed using data from wild type and heterozygous *Nrcam* knockout mice. 7B: Locomotion in every 10 min was plotted of different groups of mice with each treatment.

Figure 8.

PLG treatment and novelty seeking, sociability and marble burying behavior

8A: Difference in time spent near the novel object compared to the familiar object.

Differences of time of rearing towards the novel and familiar objects were shown. 8B:

Difference of time spent in the compartment containing the unfamiliar target mouse to

observe social preferences. 8C: Mean $\pm$ the standard error of the mean for the number of

burying marbles were plotted. For all, sample size shown in parenthesis. Data are expressed

as mean $\pm$ the standard error of the mean.

Figure 9.

Anxiety traits and PLG

Anxiety observed in the Zero Maze was shown. Vertical axis shows percentage time spent in the open section of the zero maze. Sample size shown in parenthesis. Data are expressed as mean $\pm$ the standard error of the mean. * Significant difference from wild type mice. (Fisher's PLSD, $P < 0.05$).

Figure 10.

Alcohol Preference in C57BL/6JJmsSLC mice treated with PLG

C57BL/6JJmsSLC mice were treated with saline or PLG injection i.p. daily, and were compared for consumption of ethanol. Data are expressed as mean $\pm$ the standard error of the mean of alcohol consumption from 16% alcohol by drinking in seven days.