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A spontaneous deletion of α -synuclein is associated with an increase in CB1 mRNA transcript and receptor expression in the hippocampus and amygdala: effects on alcohol consumption

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Abstract

α -Synuclein (α -syn) protein and endocannabinoid CB1 receptors are primarily located in presynaptic terminals. An association between α -syn and CB1 receptors has recently been established in Parkinson's disease, but it is completely unknown whether there is an association between these two proteins in alcohol addiction. Therefore, we aimed to examine the α -syn mRNA transcript and protein expression levels in the prefrontal cortex, striatum, amygdala and hippocampus. These brain regions are the most frequently implicated in alcohol and other drug addiction. In these studies, we used C57BL/6 mice carrying a spontaneous deletion of the α -syn gene (C57BL/6^{S α ca}−/−) and their respective controls (C57BL/6^{S α ca}+/+). These animals were monitored for spontaneous alcohol consumption (3–10%) and their response to a hypnotic-sedative dose of alcohol (3 g/kg) was also assessed. Compared with the C57BL/6^{S α ca}+/+ mice, we found that the C57BL/6^{S α ca}−/− mice exhibited a higher expression level of the CB1 mRNA transcript and CB1 receptor in the hippocampus and amygdala. Furthermore, C57BL/6^{S α ca}−/− mice showed an increase in alcohol consumption when offered a 10% alcohol solution. There was no significant difference in sleep time after the injection of 3 g/kg alcohol. These results are the first to reveal an association between α -syn and the CB1 receptor in the brain regions that are most frequently implicated in alcohol and other drug addictions.

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Conflict of interests

None

Keywords

Cannabinoid; C57BL/6 mice; genetic expression; spontaneous mutation; addiction

INTRODUCTION

α -Synuclein (α -syn) is a small acidic protein that is widely expressed throughout the brain, and its expression is primarily concentrated near the synaptic vesicles in the presynaptic axon terminals (Leong et al., 2009; Waxman and Giasson, 2009). One of the primary functions of α -syn is thought to be the modulation of synaptic neurotransmission. It has been shown that α -syn participates in the correct folding of synaptic SNAREs and the release of the neurotransmitters dopamine and glutamate (Chua and Tang, 2006; Gureviciene et al., 2007; Senior et al., 2008). Although α -syn is best known for its relation with synucleinopathies, which are a diverse group of neurodegenerative disorders associated with incorrectly folded α -syn (Jellinger, 2003; Uversky, 2008), many studies have shown that there is an association between α -syn and alcohol consumption in many species, including mice (Ziolkowska et al., 2008), rats (Liang and Carr, 2006), primates (Walker and Grant, 2006), and humans (Bönsch et al., 2004, 2005a).

The cannabinoid receptor CB1 and the fatty acid amide hydrolase enzyme (FAAH), which is responsible for the hydrolysis of endocannabinoid ligands (Piomelli et al., 2006), are two of the major components of the endocannabinoid system in the brain. Similar to the α -syn protein, most of the functional CB1 receptors are located in the presynaptic nerve terminals, and the major function of CB1 receptors is to control the release of neurotransmitters, such as dopamine, GABA and glutamate (for review, see López-Moreno et al., 2008). Another similarity with α -syn is that CB1 also appears to control alcohol consumption. Previous studies have shown that blocking CB1 causes a reduction in alcohol consumption and other alcohol-related behaviours in many rodent laboratory models (e.g., López-Moreno et al., 2007; Vinod et al., 2008); however, these results are less conclusive in humans (Soyka et al., 2008; Maccioni et al., 2010). One significant difference between α -syn and CB1 is their distribution patterns of mRNA expression in the brain. Although α -syn and CB1 are abundant in the prefrontal cortex, hippocampus and amygdala, the striatum exhibits a low mRNA expression of α -syn and a high mRNA expression of CB1 receptors (Allen Mouse Brain Atlas).

To the best of our knowledge, only three original studies have been published that investigate the relation between α -syn and CB1 receptors. These studies have shown that administering a cannabinoid receptor agonist inhibits the proteasomal synthase inhibitor-induced accumulation of α -syn in vitro (Jeon et al., 2011). Furthermore, it has been shown that the overexpression of an A53T missense mutation in the α -syn protein (Kurz et al., 2010) and deletion of the α -syn gene (García-Arencibia et al., 2009) are associated with a reduced level of CB1 transcript and a reduction in receptor binding in mouse models of Parkinson's disease.

Based on this study by García-Arencibia et al., (2009), in which the CB1 transcript expression pattern was altered in the striatum of an α -syn knockout mouse model, we decided to explore the mRNA transcript expression levels of *Cnr1* and *Faah* gene in the striatum, prefrontal cortex, amygdala and hippocampus of an α -syn knockout mouse. These four brain areas were chosen because they are the areas most frequently implicated in alcohol addiction. We used a specific C57BL/6 mouse substrain (OlaHsd) that carries a spontaneous deletion of the α -syn gene (*Snca*) that results in the complete absence of the α -syn mRNA and protein (Specht and Schoepfer, 2001) and their respective controls.

Furthermore, we also aimed to evaluate the association between spontaneous mRNA patterns and innate alcohol consumption in these mice.

MATERIALS AND METHODS

Subjects

A total of 80 C57BL/6 OlaHsd (C57BL/6^{*Snca*^{-/-}}) and 80 C57BL/6 (C57BL/6^{*Snca*^{+/+}}) male mice were used in the following experiments: 6 mice were used to determine the α -syn genotype and 24 mice were used in the gene expression experiment (experiment 1), 12 mice were used in the CB1 protein expression experiment (experiment 2), 88 mice were used in the alcohol/saccharin consumption experiment (experiment 3), and 30 mice were used in the loss of righting reflex (LORR) experiment (experiment 4). All of the mice were ordered from Harlan Laboratories (Barcelona, Spain) at 5–7 weeks of age. Food was available *ad libitum* during all of the protocols. All of the mice were housed according to their group (6–8 mice) except for the 88 mice in the alcohol/saccharin consumption experiment, which were individually housed. The mice were housed in a room with a controlled light/dark period (lights off at 12:00 A.M.). All of the procedures were conducted in strict compliance with the European Community Council Directive (86/609/EEC) and the Animal Welfare Committee of the Universidad Complutense de Madrid.

The spontaneous deletion of *Snca*

The C57BL/6J OlaHsd mice (*Snca*^{-/-}) carry a spontaneous deletion of the α -syn gene (Specht and Schoepfer, 2001). The origin of this deletion likely arises because of a kind of “founder effect” as a result of the establishment of a new population of mice in a laboratory. One clear example of this is the segregation of C57BL/6 substrains between 1948 and 1951 when the mice were sent to Jackson Laboratories (C57BL/6J) or the National Institutes of Health (e.g., C57BL/6N). The spontaneous *Snca* deletion was first described by Specht and Schoepfer (2001) and was accidentally discovered during a study using a wide scale mRNA expression profiling cDNA array. Specht and Schoepfer discovered that the *Snca* deletion occurred exclusively in the OlaHsd substrain (C57BL/6^{*Snca*^{-/-}}), which is a well-established colony at Harlan Laboratories (Bicester, UK). Harlan regularly provides these mice and other mouse substrains that exhibit normal expression of α -syn (C57BL/6^{*Snca*^{+/+}}), which were used in the present experiments.

α -Syn genotype determination

The presence of the α -syn locus (*Snca*) was analysed in the C57BL/6 OlaHsd (C57BL/6^{*Snca*^{-/-}}) and C57BL/6 (C57BL/6^{*Snca*^{+/+}}) mice. Genomic DNA was isolated from the liver using a DNeasy Blood and Tissue Kit (Qiagen Iberia, Spain). Polymerase chain reaction (PCR) was performed using 1 μ g of genomic DNA as a template. PCR cycling included denaturation at 94 °C (30 s), annealing at 52 °C (30 s), and extension at 72 °C (30 s) for a total of 35 cycles. Specific primers were used to amplify the mouse *Snca* exon 6 (AAGACTATGAGCCTGAAGCCTAAG and AGTGTGAAGCCACAACAATATCC; 266-bp fragment) (Chen et al., 2002) and the deletion junction, termed D6Slab17, which spans the deletion Del(6)*Snca*1Slab (TTGATAGTTCCACTGTTCTGGC and GTAACAATACAGCAAGAGATAC; 179-bp fragment) (Specht and Schoepfer, 2004). PCR products were resolved on a 3% agarose gel and verified by sequencing. Genomic PCR experiments confirmed the absence of the α -syn locus in the C57BL/6^{*Snca*^{-/-}} mice and its presence in the C57BL/6^{*Snca*^{+/+}} mice. In contrast to the C57BL/6^{*Snca*^{-/-}} mice, amplification of the genomic DNA fragment corresponding to the deletion-spanning locus D6Slab17 did not yield a PCR product in the C57BL/6^{*Snca*^{+/+}} mice. The identity of each PCR product was confirmed by sequencing. The results confirmed that the C57BL/6^{*Snca*^{-/-}} mice carried a chromosomal deletion of the α -syn gene locus.

Experiment 1: Determination of specific mRNA transcript levels

The mRNA transcript expression levels in the C57BL/6^{Snca}^{-/-} and C57BL/6^{Snca}^{+/+} mice were assessed after the mice had acclimated to our colony for one week. Briefly, the brains of the mice were removed from their skulls, and the prefrontal cortex, striatum, amygdala and hippocampus were quickly dissected on ice, immediately frozen on dry ice and stored at -80 °C. Total RNA was isolated from these four brain regions using TRIzol reagent (Invitrogen) and stored at -80 °C. For the experiment, 5 µg of total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems) and TaqMan Gene® Expression Assays (Applied Biosystems). All of the assays that were performed were designed by Applied Biosystems. A series of 10-fold dilutions of each gene was used to validate the efficiency of each TaqMan probe and to confirm that the target and reference gene efficiencies were comparable (indicated by a near-zero slope value from the target and reference genes). Mouse *Dnaja8* was used as a reference to normalise all of the other mRNAs, and the C57BL/6^{Snca}^{+/+} mice were used for calibration. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) and Schmittgen and Livak (2008).

Experiment 2: Immunohistochemistry analysis

The mice were anaesthetised with pentobarbital (50 mg/kg, i.p.) and perfused with 4% paraformaldehyde dissolved in 0.1M phosphate buffer (PB, pH 7.4). After perfusion, the brains were dissected and immersed overnight in the same fixative solution. Coronal brain sections (30 µm) were obtained using a slicing vibratome (Leica, Madrid, Spain) and kept in PB with 0.02% sodium azide solution at 4 °C until use. Immunostaining was carried out on free-floating sections with a standard avidin-biotin immunohistochemical protocol as previously described (Granado et al., 2010). Sections were incubated in 3% H₂O₂ in PBST (Phosphate Buffered Saline containing 0.1% triton X-100) for 10 min to inactivate endogenous peroxidase activity and pre-blocked by incubation with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were incubated overnight at 4 °C with polyclonal anti-CB1 receptor antibody (dilution 1:1000; generously provided by Dr. Ken Mackie, University of Washington, Seattle, USA) in a solution of PBST containing normal goat serum and sodium azide. After careful washing, the sections were incubated with biotinylated secondary antisera (Vector) at room temperature and developed using diaminobenzidine (DAB). The reaction was monitored every 5 min using an optical microscope (Leica). After washing three times for 10 min at room temperature, the sections were mounted on gelatin-coated slides, air-dried, dehydrated in ascending concentrations of alcohol, cleared with xylene and covered with coverslips under Permount. Quantification of CB1 expression was performed using an image analysis system (Analytical Imaging Station (AIS), Imaging Research Inc., Linton, UK) and a 5x lens. Colour intensities were converted into a grey scale. We quantified the stained area in the C57BL/6^{Snca}^{-/-} and C57BL/6^{Snca}^{+/+} mice as a proportion of the total scanned area, and refer to it as the proportional stained area (Granado et al., 2008; Ares-Santos et al., 2012). The measurements were carried out in groups consisting of six mice with 5–6 sections/mouse in the prefrontal cortex, hippocampus, striatum and amygdala. For the quantifications, the dorsal region of the hippocampus was defined using Ammon's horn and the dentate gyrus. The striatum was defined using the mid-rostral sections, and the amygdala quantifications included the lateral, basolateral and basomedial nuclei.

Experiment 3: Analysis of alcohol/saccharin consumption (two-bottle choice protocol)

Two-bottle choice home cage consumption and preference were evaluated during a 24 h period using a modified version of the protocol described by Basavarajappa et al. (2006).

Forty-four mice (half C57BL/6^{Snca^{-/-}} and half C57BL/6^{Snca^{+/+}}), individually housed, were offered alcohol in 50-mL bottles. The alcohol concentration in the bottles varied according to the following timeline: a 3% (v/v) alcohol solution was provided for the first four days, a 6% solution was provided for the next four days, and a 10% solution was provided for the next 12 days. All of the mice had access to a bottle of water *ad libitum* throughout the entire experiment. The positions of the bottles were changed every day, and the bottles were refilled with fresh solution every four days. An average of four bottles were placed in empty cages and used as a control to determine the loss of alcohol resulting from evaporation. The data are expressed as grams of alcohol consumed per kilogram of body weight. Another 44 mice (half C57BL/6^{Snca^{-/-}} and half C57BL/6^{Snca^{+/+}}) were offered a 0.01% saccharin solution under the same experimental conditions.

Experiment 4: Analysis of the Loss of Righting Reflex (LORR)

Thirty mice (half C57BL/6^{Snca^{-/-}} and half C57BL/6^{Snca^{+/+}}) were injected with alcohol (3 g/kg, i.p.), and the length of alcohol-induced LORR (sleep time) was measured as previously described by Blednov et al. (2001). Latency to LORR was defined as the period of time (seconds) between the alcohol injection and LORR. LORR was defined as the inability of a mouse to right itself within 30 s. Recovery of the righting response was defined as the ability of a mouse to right itself twice in 1 min. The dose of alcohol (3 g/kg, i.p.) was chosen because it has been previously used with success to evaluate and discriminate the acute functional tolerance to alcohol in eight inbred strains of mice (Ponomarev and Crabbe, 2002).

Drugs

An alcohol solution was prepared for consumption from a stock solution of 99% alcohol (Panreac, S.A. Barcelona, Spain).

Statistical analysis

For the analyses of mRNA transcript expression, a two-way ANOVA with the type of mouse and the brain region as the between-groups factors was performed (experiment 1, Table 1). Similarly, CB1 receptor expression was analysed using a two-way ANOVA (experiment 2, Figure 1). The data regarding alcohol/saccharin consumption in the 2-bottle choice protocol was analysed using a two-way ANOVA with the day number as the within-subject factor and the type of mouse as the between-groups factor (experiment 3, Figure 2). The LORR data were analysed using Student's *t* test (experiment 4, Figure 3). If significant ANOVA results were obtained, a post hoc Tukey's honestly significant difference (HSD) test was performed to determine which between-group factors were significantly different. The SPSS statistical software package (version 17.0) for Windows (Chicago, IL) was used for all of the statistical analyses.

RESULTS

Experiment 1: Analysis of the spontaneous differences in mRNA transcript levels in the C57BL/6^{Snca^{-/-}} mice

The goal of the first experiment was to analyse changes in the mRNA transcript expression levels of two main components of the endocannabinoid system (the CB1 receptor and the FAAH enzyme) in mice with a spontaneous deletion of the α -syn gene. We focused on four brain areas, the prefrontal cortex, striatum, amygdala and hippocampus, because of their association with alcohol and drug addiction. The relative changes in gene expression in the C57BL/6^{Snca^{-/-}} mice compared with the C57BL/6^{Snca^{+/+}} mice are represented as a percentage of the difference (Table 1). The C57BL/6^{Snca^{-/-}} mice showed a significant

increase in *Cnr1* expression in the amygdala and hippocampus compared with the C57BL/6^{*Snca*^{+/+}} mice (two-way ANOVA: type of mouse, $F_{(1,84)}=12.99$, $P<.001$; region, $F_{(3,84)}=3.20$, $P<.05$; type of mouse $\times$ region, $F_{(3,84)}=3.20$, $P<.05$). There was no significant difference in the *Faah* mRNA expression levels between these two groups of mice in any of the brain regions examined. *Snca* expression was not detected in any of the brain regions.

Experiment 2: Analysis of the spontaneous difference in the CB1 protein expression level in C57BL/6^{*Snca*^{-/-}} mice

The increase in *Cnr1* mRNA expression that we observed in the C57BL/6^{*Snca*^{-/-}} mice in the first experiment suggests that CB1 receptors may be one of the most significantly affected components of the cannabinoid system associated with the spontaneous deletion of *Snca*. This result prompted us to conduct our second experiment in which we analysed the CB1 protein expression level in these same four brain regions. As expected, the C57BL/6^{*Snca*^{-/-}} mice showed a higher expression level of CB1 receptor in the hippocampus and amygdala compared with the C57BL/6^{*Snca*^{+/+}} mice (Figure 1a), which is consistent with their transcript expression levels. Furthermore, both types of mice showed that the highest concentration of CB1 receptor expression was found in the hippocampus compared with the amygdala, striatum and prefrontal cortex (two-way ANOVA: type of mouse, $F_{(1,210)}=76.15$, $P<.0001$; region, $F_{(3,210)}=63.6$, $P<.0001$; type of mouse $\times$ region, $F_{(3,210)}=20.86$, $P<.0001$).

Experiment 3: Analysis of alcohol/saccharin consumption (two-bottle choice protocol) in the C57BL/6^{*Snca*^{-/-}} mice

Because the α -syn protein and CB1 receptor have been repeatedly implicated in affecting the alcohol consumption in many different species, we expected that differences in the gene and protein expression patterns would be associated with different patterns of alcohol consumption. Therefore, we investigated alcohol consumption at increasing concentrations (3, 6 and 10%) in the C57BL/6^{*Snca*^{-/-}} and C57BL/6^{*Snca*^{+/+}} mice. Furthermore, we also tested whether the expected effects on alcohol consumption are specific to the alcohol solution or the result of a natural reward system by including groups of mice that drank a saccharin solution instead of an alcohol solution. Figure 2a shows the time course of alcohol consumption in grams per kilogram of body weight using the two-bottle choice paradigm. Each point represents the mean of two measurements taken every 24 h. Significant differences between the two types of mice appeared after the introduction of a 10% alcohol solution (two-way ANOVA, mice $F_{(1,40)}=120.60$, $P<.0001$; days $F_{(5,200)}=13.87$, $P<.0001$; mice $\times$ days $F_{(5,200)}=5.28$, $P<.001$). On average, the C57BL/6^{*Snca*^{-/-}} mice drank twice as much alcohol as the C57BL/6^{*Snca*^{+/+}} mice. The total amount of alcohol consumption during the 12 days of exposure to the 10% alcohol solution is shown in Figure 2b. The C57BL/6^{*Snca*^{-/-}} mice consumed an average of 81.6 g/kg and the C57BL/6^{*Snca*^{+/+}} mice consumed an average of 41.1 g/kg. Figure 2c shows the time course of water intake, which was the specular image of alcohol consumption. There was no significant difference in the consumption of the saccharin solution or water in the saccharin group (Figure 2d-f). The total amount of saccharin consumption during the last 12 days of exposure to 0.01% saccharin was an average of 39.6 mL in the C57BL/6^{*Snca*^{-/-}} mouse group and 37.4 mL in the C57BL/6^{*Snca*^{+/+}} mouse group.

The results and significant differences between the groups for alcohol preference paralleled the alcohol consumption results. These results showed that C57BL/6^{*Snca*^{-/-}} mice have an alcohol preference level that was around 60–65% and that the C57BL/6^{*Snca*^{+/+}} mice only have a preference level of 32–38%. No differences in saccharine preference (~80%), total fluid intake, or total food intake were observed between these two types of mice.

Experiment 4: Loss of righting reflex (LORR) following a hypnotic-sedative dose of alcohol (3 g/kg) in C57BL/6^{*Snca*^{-/-}} mice

In the fourth and final experiment, we investigated whether the spontaneous deletion of *Snca* could alter the effects of a hypnotic-sedative dose of alcohol. Because it is extremely difficult to investigate the consequences of a high hypnotic-sedative dose of alcohol using the two-bottle choice protocol, we decided to inject the mice with a high dose of alcohol (3 g/kg, i.p.). Our results showed that there was no significant difference between the C57BL/6^{*Snca*^{-/-}} and C57BL/6^{*Snca*^{+/+}} mice in the latency to LORR (*t* test, *P*=.73) or the duration of LORR (sleep time) (*t* test, *P*=.17) after alcohol injection (Figure 3).

DISCUSSION

This study provides evidence that supports an association between the α -syn protein and the CB1 receptor. We used a specific mouse substrain (C57BL/6J OlaHsd, ^{*Snca*^{-/-}}) that carries a spontaneous deletion of the α -syn gene (Specht and Schoepfer, 2001) that we have previously confirmed using the Illumina's Mouse MD Linkage Panel (Zurita et al., 2010). In addition to α -syn, it has also been shown that the multimerin-1 gene (*Mmrn1*) has also been deleted in this mouse substrain (Specht and Schoepfer, 2004). However, the *Mmrn1* gene encodes a soluble protein that has been found in platelets and the endothelium but not in the brain (Jeimy et al., 2008), which makes it very unlikely that the biochemical and behavioural results presented in this study are the consequence of a lack of the multimerin-1 protein. Currently, the C57BL/6J OlaHsd mice (^{*Snca*^{-/-}}) have been used to successfully study the role of α -syn in several facets, including impulsivity (Peña-Oliver et al., 2012), neuromuscular pathology (Pelkonen and Yavich, 2011), a model of prion disease (Asuni et al., 2010) and beta-adducin protein function (Gajovic et al., 2006). In the present study, we used these spontaneously mutant mice to test the hypothesis that *Snca* deletion affects CB1 mRNA transcript and receptor expression and alcohol consumption.

CB1 mRNA transcript and protein expression in C57BL/6^{*Snca*^{-/-}} mice

Previous studies have demonstrated that adaptive compensatory mechanisms after genetic overexpression or genetic deletion of α -syn in mice (Cabeza-Arvelaiz et al., 2011; Chadchankar et al., 2011). However, only two previous studies have explored the changes in CB1 mRNA transcript and receptor expression after the genetic manipulation of *Snca* expression. Both of these studies were performed in murine models of Parkinson's disease. One of these studies used an engineered α -syn KO mouse and showed that there was a decrease in the CB1 transcript and protein expression levels in different subregions of the mouse striatum (García-Arencibia et al., 2009). The other study reported a similar reduction in CB1 mRNA transcript and protein expression levels in the striatal subregions, but this reduction was only observed in one of the two lines of mice overexpressing the human A53T missense mutation of the α -syn protein (Kurz et al., 2010). In the present study, however, we did not find any significant change in the CB1 mRNA transcript or protein expression levels in the striatum. This discrepancy between the results from these studies may be partially explained by the age of the mice. In the previous studies older mice (i.e., between 12 and 18 months) were used, but in this study the mice were approximately 3 months old. Previous studies have shown that CB1 receptor density (Canas et al., 2009) and functionality (Wang et al., 2003) decrease with age. Furthermore, a direct comparison of the results from these earlier studies and the current study cannot be made because the mice are not in the same genetic background. The spontaneous deletion of *Snca* originated and was maintained in the inbred C57BL/6 background, but the mice in the García-Arencibia et al. and Kurz et al. studies were generated in the 129/SvEvTac and FVB/N backgrounds, respectively. A dramatic example that emphasises the effects of different genetic inbred background strains was the generation of CB1-null mice in the C57BL/6 and CD1

backgrounds. There are contrasting patterns in bone mass regulation in these two different genetic backgrounds (Tam et al., 2006).

Another point to be considered is the probability that transcription and translation of the CB1 receptor in the cell body would be affected by the high expression in the axon terminals of the CB1 receptor and α -syn protein. Devised new experiments investigating how the blockade of the retrograde transport from axon terminals to the soma would provide the answer upon this point. Until now, it has been shown that alpha-synuclein regulates proteins intended for anterograde (members of kinesin family: KIF1A, KIF1B, KIF2A and KIF3A,) and retrograde axonal transport (dynein, dynamin and dynactin1) (Chung et al., 2009).

According to the Allen Mouse Brain Atlas (2009), which used non-mutant C57BL/6 mice, the prefrontal cortex, hippocampus and amygdala (at coronal positions 9625 and 5225) show the highest mRNA expression levels of *Snca* and the striatum shows the lowest expression level (at coronal position 7625). Because the CB1 and α -syn protein are highly expressed in the presynaptic terminals, we hypothesise that the biological compensatory effects (e.g., in CB1 mRNA transcript and protein expression levels) resulting from the absence of *Snca* expression would occur in the areas with the highest *Snca* expression levels. Specifically, our results agree with the biochemical hypothesis that critical neuroadaptations occur in the endocannabinoid system in two of the brain regions that are frequently implicated in alcoholism and drug addiction (i.e., the hippocampus and amygdala).

Alcohol consumption, α -syn and CB1 receptors

The C57BL/6^{Snca^{-/-}} mice exhibited greater alcohol consumption than the C57BL/6^{Snca^{+/+}} mice when offered a 10% alcohol solution. We must assume that this finding is associated with innate differences between these types of mice for alcohol consumption because we did not evaluate the effect of alcohol consumption on the α -syn or CB1 mRNA transcript and protein expression levels. The deletion of *Snca* has been widely studied in the context of synucleinopathies (e.g., Parkinson's disease and multiple system atrophy), but very few studies have examined *Snca* deletion in the context of drug addiction. To assist in the interpretation of our results, we needed to know the association between α -syn and alcohol. Interestingly, after alcohol withdrawal in C57BL/6 mice, there is an increase in α -syn mRNA transcript expression in the blood and an increase in the α -syn protein level in the amygdala but not in the striatum (Ziolkowska et al., 2008). Other studies using inbred alcohol-preferring and non-preferring rats and different genetic approaches indicate that there is a genetic link between alcohol addiction and the *Snca* locus (Spence et al., 2005, 2009; Liang and Carr, 2006). Studies have shown that there is an increase in α -syn mRNA transcript and protein expression in the hippocampus of inbred alcohol-preferring rats compared with non-preferring rats (Liang et al., 2003). Furthermore, it has been shown that the α -syn mRNA transcript expression level is higher in the blood of cynomolgus monkeys after long-term chronic alcohol self-administration (Walker and Grant, 2006). Although single nucleotide polymorphism studies across the SNCA gene have only shown nominally significant associations with alcohol dependence and alcohol craving in humans (Foroud et al., 2007; Clarimon et al., 2007), alcohol use disorders and dependence have been associated with the promoter of the α -syn gene, REP1, independent of Parkinson's disease (Bönsch et al., 2005b; Brighina et al., 2009). In addition, an increase in the α -syn mRNA transcript and protein expression in the peripheral blood of patients with alcoholism, craving and impulsivity have also been reported (Bönsch et al., 2004, 2005a). Taken together, there appears to be an increase in α -syn mRNA transcript and protein expression in selectively bred alcohol-preferring rats and after chronic alcohol consumption and alcohol withdrawal across different species.

In regard to CB1, an association between increased CB1 mRNA transcript and protein expression and a higher alcohol consumption is to be expected because mice lacking the CB1 receptor show a lower preference for alcohol (Lallemant and de Witte, 2004), a reduced conditioned place preference (Houchi et al., 2005; Thanos et al., 2005), a decrease in alcohol self-administration, an increase in alcohol sensitivity (Naassila et al., 2004; Thanos et al., 2005), and a lack of alcohol-induced dopamine release in the nucleus accumbens (Hungund et al., 2003).

In most animal models of alcohol self-administration, it is virtually impossible to investigate the effects of a high hypnotic-sedative dose of alcohol (acute intoxication). We believe, however, that these effects are an important issue to study because acute alcohol intoxication and even ethyl comas are frequently observed in humans. We did not observe a significant difference in the duration of LORR between the two types of mice. This lack of effect on the LORR phenotype may be the result of a behavioural ceiling effect at the 3 g/kg alcohol dose. A future set of experiments that establish a gradient between alcohol self-administration and acute alcohol intoxication should be performed. Finally, we did not find any significant difference in saccharine intake. Both types of mice showed similar patterns in saccharine consumption. This result suggests that the hedonic drive for a natural reward, at least evaluated with saccharine, is not affected by the changes in the CB1 receptor mRNA and protein expression levels observed in this study.

CONCLUSIONS

The present study provides convincing preliminary evidence that supports an association between α -syn and CB1 in the brain regions that are most frequently implicated in drug addiction and alcohol consumption. The spontaneous deletion of *Snca* is associated with an increase in CB1 mRNA transcript and protein expression in the hippocampus and amygdala and is also associated with an initial preference for alcohol consumption. Further studies are needed to determine the precise functional and neuroanatomical association between these two highly expressed presynaptic proteins. α -syn and CB1 receptor appear to be promising biological markers for alcohol addiction across species, and future studies should investigate the role of α -syn and CB1 receptor in the long-term maintenance of alcohol consumption and other parameters of alcohol addiction.

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Figure 1. Higher CB1 receptor expression levels in the hippocampus and amygdala of C57BL/6Snca^{-/-} mice compared with C57BL/6Snca^{+/+} mice

Photomicrographs of brain sections stained with a CB1 receptor antibody from C57BL/6Snca^{+/+} and C57BL/6Snca^{-/-} mice. Histograms (a) show the proportional stained area of CB1 receptor immunoreactivity in different brain areas. ** $P < 0.01$, C57BL/6Snca^{-/-} mice compared with C57BL/6Snca^{+/+} mice and ## $P < 0.01$ and && $P < 0.01$, compared with hippocampus. The data are expressed as the mean $\pm$ SEM ($n = 6$ per group). Panels b-g show representative immunohistochemical images of CB1 receptor expression in the hippocampus (b and c), amygdala (d and e), striatum (f) and prefrontal cortex (g) in both types of mice.

The scale bar represents 500 μm in panels b-g and 40 μm in the bottom panels b-e (40x magnification). PFC=prefrontal cortex; Hip=hippocampus; Str=striatum; Amy=amygdala.

Figure 2. The C57BL/6^{Snca}^{-/-} mice exhibit greater alcohol consumption than the C57BL/6^{Snca}^{+/+} mice

The mice (n=44 for the alcohol group, and n=44 for the saccharin group) were given daily access to two types of bottles, one with water and the other with either alcohol or saccharin, *ad libitum*. Each point represents the mean $\pm$ SEM of two measurements taken every 24 h. The mice were exposed to an initial concentration of 3% alcohol, and the concentration was increased every 4 days up to a final concentration of 10%, which was maintained for 12 days (panel a). The total alcohol intake during those 12 days is shown in panel b, and significant differences were observed between the mouse groups; *** P <.001. Daily water intake is shown in panel c. Daily saccharin and water consumption is shown in panels d and f, and the total amount of saccharin intake for the last 12 days is shown in panel e. No differences were observed in saccharin consumption between the mouse groups at the concentration tested (0.01%).

■ C57BL/6^{Snca^{-/-}}
□ C57BL/6^{Snca^{+/+}}

Figure 3. No difference between the C57BL/6^{Snca^{-/-}} and C57BL/6^{Snca^{+/+}} mice after a hypnotic-sedative dose of alcohol

There were no significant differences between the two types of mice in either latency to alcohol-induced loss of righting reflex (LORR) (panel a) or duration of LORR (sleep time) (panel b) after an i.p. injection of 3 g/kg alcohol. The data are expressed as the mean $\pm$ SEM (n=15 per group).

Table 1

Differences in the gene expression levels between the C57BL/6^{Sncα^{-/-}} and C57BL/6^{Sncα^{+/+}} mice in the prefrontal cortex, striatum, amygdala and hippocampus. Data represent the relative gene expression levels in the C57BL/6^{Sncα^{-/-}} mice compared with the C57BL/6^{Sncα^{+/+}} mice and are shown as percentages.

	Brain region			
	Prefrontal	Striatum	Amygdala	Hippocampus
<i>Snea</i>	0.0(±0)	0.0(±0)	0.0(±0)	0.0(±0)
<i>Chn1</i>	108.1(±9.1)	98.3(±6.5)	128.1(±4.3)**	134.7(±4.9)**
<i>Faah</i>	112.2(±4.9)	113.8(±10.3)	114.5(±6.1)	94.5(±5.3)

* $P<.05$ and
** $P<.01$.

The *Snea* gene encodes the alpha-synuclein protein and was not detected in any of the regions studied in the C57BL/6^{Sncα^{-/-}} mice. The *Chn1* gene encodes the cannabinoid receptor CB1. The *Faah* gene encodes the fatty acid amide hydrolase enzyme.