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The role of cannabinoid 1 receptor expressing interneurons in behavior

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ABSTRACT

Schizophrenia is a devastating neurodevelopmental disorder that affects approximately 1% of the population. Reduced expression of the 67-kDa protein isoform of glutamic acid decarboxylase (GAD67) is a hallmark of the disease and is encoded by the *GAD1* gene. In schizophrenia, GAD67 downregulation occurs in multiple interneuronal subpopulations, including the *cannabinoid receptor type 1* positive (CNR1 +) cells, but the functional consequences of these disturbances are not well understood. To investigate the role of the CNR1-positive GABAergic interneurons in behavioral and molecular processes, we employed a novel, miRNA-mediated transgenic mouse approach. We silenced the *Gad1* transcript using a miRNA engineered to specifically target *Gad1* mRNA under the control of *Cnr1* bacterial artificial chromosome. Behavioral characterization of our transgenic mice showed elevated and persistent conditioned fear associated with an auditory cue and a significantly altered response to an amphetamine challenge. These deficits could not be attributed to sensory deficits or changes in baseline learning and memory. Furthermore, HPLC analyses revealed that *Cnr1*/*Gad1* mice have enhanced serotonin levels, but not dopamine levels in response to amphetamine. Our findings demonstrate that dysfunction of a small subset of interneurons can have a profound effect on behavior and that the GABA-ergic, monoamine, and cannabinoid systems are functionally interconnected. The results also suggest that understanding the function of various interneuronal subclasses might be essential to develop knowledge-based treatment strategies for various mental disorders including schizophrenia and substance abuse.

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Introduction

Genetic and pharmacological studies have demonstrated that endocannabinoids (eCBs) and drugs targeting the eCB system can affect neuronal development and differentiation (Galve-Roperh et al., 2008; Harkany et al., 2007). Several recent epidemiological studies have associated increased psychotic episodes and a higher probability to develop schizophrenia as a result of adolescent cannabis abuse (Henquet et al., 2005; Matheson et al., 2011; Muller-Vahl and Emrich, 2008; van Os et al., 2002; Veen et al., 2004) Furthermore, cannabis abuse can also induce acute psychosis (Morrison et al., 2009).

The eCB system regulates emotion, stress, memory and cognition. The eCB N-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized and released postsynaptically to act as retrograde messengers at presynaptic cannabinoid receptor 1 (CNR1, also known as CB1R or CB1A) located on both glutamatergic and gamma-aminobutyric acid (GABA)-ergic axon terminals (Katona et al., 2001, 2006). They suppress neurotransmitter release (Wilson et al., 2001) and are very effective modulators of synaptic plasticity (Katona and Freund, 2012). In the adult brain, CNR1 is expressed at high levels in the neocortex, hippocampus, striatum, amygdala and cerebellum (Pettit et al., 1998; Tsou et al., 1998). On GABAergic interneurons, CNR1 is primarily expressed on basket cells and represents a smaller subpopulation of cholecystokinin (CCK) expressing interneurons (Eggan et al., 2010a; Katona et al., 2000, 2001). They are slowly adapting and are coupled to 3–8 Hz theta oscillations (Klausberger and Somogyi, 2008).

Previous animal studies have focused on genetic inactivation of *Cnr1* receptors. These animals showed increased mortality, hypoactivity, hypoalgesia (Zimmer et al., 1999), as well as elevated arousal/anxiety that might promote enhanced social discrimination memory (Litvin et al., 2013; Martin et al., 2002). However, the majority of these

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experiments were not able to differentiate between the CNR1 effects mediated through glutamatergic and GABAergic terminals. To directly address the role of inhibitory interneurons in endocannabinoid circuitry, we silenced *Gad1*, the gene encoding the primary enzyme responsible for producing GABA in the brain, using a *Cnr1* bacterial artificial chromosome (BAC) driven system in transgenic mice. After validating the expression specificity and efficacy, we performed comprehensive behavioral and neurochemical assessments of these animals. These experiments demonstrate the importance of *Cnr1*+ GABAergic interneuron population and provide insight into the specific role of cannabinoid systems in inhibitory circuitry.

Materials and methods

Mouse generation

RP24-370M5 BAC, containing the mouse *cannabinoid receptor1* (m*Cnr1*) locus (Chr4: 33,837,634–33,989,366, NCBI Build 38.1), was purchased from the BACPAC Resource Center at the Children's Hospital of Oakland Research Institute (http://bacpac.chori.org/). The m*Cnr1* gene itself is mapped at Chr4: 33,924,632–33,948,831, + strand. The BAC was isolated from the original DH10B *Escherichia coli* strain via standard alkaline lysis protocol (available upon request) and transformed into EL250 *E. coli* cells (kind gift of Dr. Neil Copeland, NCI). EL250 cells were instrumental for our BAC modifications as they are capable of heat-inducible expression of recombination proteins and arabinose-inducible FLP recombination. The presence of the *mCnr1* locus in RP24-370M5 was verified by restriction enzyme digest mapping. A BAC targeting construct was generated using previously engineered targeting constructs for the *mNPY* gene, described in detail by Garbett et al. (2010). In essence, the *mNPY* homology arms were swapped with the *mCnr1* homology arms in pSTBlue-1 plasmid vector (Novagen, Madison). The *mCnr1* targeting construct carried Cnr1 5′ (205 bp) and 3′ (260 bp) homology arms, surrounding eGFP, β-globin minigene and an FRT-flanked neomycin resistance cassette. The β-globin minigene contained a *Gad1* targeting miRNA in an intronic location (allowing the in vivo release of functional miRNA) which effectively reduced the GAD67 protein to undetectable levels in cell cultures (Garbett et al., 2010). Digestion with EcoRI released the targeting fragment from the base plasmid; the targeting fragment was then used for homologous recombination into the *mCnr1* containing BAC RP24-370M5. The resulting BACs were screened by PCR and confirmed with restriction mapping and sequence analysis for correct modifications. Finally, the *E. coli* strain containing the modified BAC was treated with arabinose to induce the expression of FLP recombinase, which removed the FRT-flanked neomycin resistance cassette. Proper recombination was confirmed with restriction mapping and sequence analysis of the region of interest. The modified RP24-370M5 BAC was isolated with alkaline lysis and purified with *Sepharose* CL-4B chromatography as described previously (Gong and Yang, 2005). Transgenic mice were generated by injection of circular modified BAC into fertilized C57Bl/6 mouse oocytes by the University of California Irvine Transgenic Mouse Facility. Transgenic founder mice were identified by PCR using construct-specific primer pairs.

Mouse genotyping

Tail samples of 2 mm were taken at P21. The tissue was digested over night at 55C in 245 μl Direct PCR (tail) (Viagen Biotech, Cat# 102-T) and 5 μl Proteinase K (Clontech, Cat# 740506), then incubated at 85C for 45 min. Primers used to genotype the samples had the following sequences: ACGACGGCAACTACAAGACC (GFP.k.geno.F1) and ACCT TGATGCCGTTCTTCTG (GFP.k.geno.R1). The annealing temperature for these primers is 60 °C (30 s), and the amplification yields a product size of 184 base pairs.

Immunohistochemistry

For immunolocation studies, animals were deeply anesthetized with isoflurane and transcardially perfused with ice cold 1 × phosphate buffer (PB) solution followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) at room temperature. All procedures were performed in accordance with the guidelines of the American Association for Laboratory Animal Science and the Vanderbilt University Institutional Animal Care. Brains were then removed and postfixed for 4 h at room temperature in 4% paraformaldehyde. Coronal sections, 40-μm thick, were prepared with a vibratome (VT1000S, Leica Microsystems, Bannockburn, IL, USA), and then washed several times in 0.1 M PB.

Brain sections were incubated for 1 h in 10% normal donkey serum in 0.1 m PB (pH 7.4). Immunostaining for eGFP was performed either with a rabbit anti-GFP (Invitrogen; 1:2000) or chicken anti-GFP (Abcam; 1:2000). Immunostaining for CNR1 was performed using 1:2000 dilution of affinity-purified polyclonal guinea pig anti-CNR1 antibody raised against the 31aa C-terminus of the mouse CNR1 (Frontier Science Co. Ltd, Hokkaido, Japan). For PV immunostaining, a 1:5000 dilution of rabbit anti-parvalbumin antiserum (Swant Ltd., Switzerland) was used. For GAD67 immunostaining, sections were pre-incubated with 70 μg ml^{-1} of a monovalent Fab fragment of donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, PA, USA) to block endogenous mouse immunoglobulins before proceeding with the standard protocol for immunolabeling with mouse anti-GAD67 (Millipore; 1:2000). The following secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used for fluorescence detection: donkey anti-guinea pig Cy3, donkey anti-rabbit AMCA, donkey anti-chicken DyLight488 and donkey anti-mouse Cy3 (all diluted 1:250). All sections were incubated with primary antibodies for 72 h at 4 °C, washed extensively and incubated in secondary antibodies for 3 h at room temperature. Immunolabeled sections were mounted in ProLong® Gold Antifade Reagent (Life Technologies, NY, USA) and examined using an Olympus BX51 fluorescence microscope with DP21 digital camera (Olympus Corporation, Tokyo, Japan). Images were stored and analyzed using ImageJ for Windows scientific imaging software (NIH, Bethesda, MD, United States) with Microscopy plugins (http://rsbweb.nih.gov/ij/). Brightness and contrast were adjusted for the whole image using Adobe Photoshop CS5 software (Adobe Systems, Inc, San Jose, CA, USA).

Animals

All animal procedures were performed according to Vanderbilt University Institutional Animal Care and Use Committee approved procedures. Male C57Bl/6J mice (3–5 months of age at start of testing), were used for all experiments. All animals were housed in groups of two to five. Food and water were available ad libitum. All mice were kept on a 12-h light–dark cycle.

Behavioral experiments

Behavioral testing was performed in the Vanderbilt Murine Neurobehavioral Laboratory. Adult male mice were handled for 5 days prior to the beginning of the battery. Prior to each testing session, mice were brought from the animal housing room into an anteroom outside each testing room and acclimated for 1 h under red light. Consecutive tests were at least 24 h apart. Experimenters were blinded to genotypes. All equipment was cleaned with Vimoba solution between animals to reduce odor contamination. Mice were evaluated behaviorally on the following tests described below in greater detail: (i) 10 min open field exploration, (ii) Irwin screen and battery of sensorimotor measures (grip strength, rotor rod and swim speed), (iii) fear conditioning, (iv) 0-maze and y-maze, (v) prepulse inhibition (PPI), (vi) social interaction and social odor investigation, and (vii) response to CNR1 agonist and amphetamine (AMPH) challenge.

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