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Features of emotional and social behavioral phenotypes of calsyntenin2 knockout mice

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ABSTRACT

Calsyntenin-2 (Clstn2) is the synaptic protein that belongs to the super family of cadherins, playing an important role in learning and memory. We recently reported that Clstn2 knockout mice (Clstn2-KO) have a deficit of GABAergic interneurons coupled with hyperactivity and deficient spatial memory. Given, that impaired functioning of GABA receptors is linked to several psychopathologies, including anxiety and autism, we sought to further characterize Clstn2-KO mice with respect to emotional and social behavior. Clstn2-KO males and females were tested in the elevated plus-maze (EPM), open field (OF), forced swim test, social affiliation and recognition test, social transmission of food preference (STFP), dyadic social interactions and marble burying test. Clstn2-KO mice demonstrated high exploration and hyperactivity in the dimly lit EPM that affect anxiety parameters. In contrast, in a more adverse situation in the OF have increased emotionality in Clstn2-KO males, not females. Assessment of hyperactivity for prolong period in the OF showed that Clstn2-KO animals were able to decline their hyperactivity, but their ambulation still remained higher than in WT littermates. Additionally, Clstn2-KO mice expressed stereotyped behavior. Strikingly, analysis of social behavior identified deficient social motivation and social recognition only in Clstn2-KO males, but not in females. Further analysis of social communication in the STFP and direct observation of agonistic interactions confirmed the reduced social behavior in Clstn2-KO males. Altogether, current results showed Clstn2 gene and sex interactions on socio-emotional performance in mice, suggesting a possible role of calsyntenin2 in psychopathological mechanisms of autism.

1. Introduction

The synapse is the structural and functional unit integrating a vast network of neurons within the brain. Accordingly, elucidating the molecular mechanisms involved in the organization and function of these synapses is essential to understanding the fundamental processes of the brain, both in normal and psychopathological states. Proteins involved in synaptic adhesion are the main molecular players to form synaptic contacts, and modulate functions of pre- and postsynaptic molecular complexes, essential features for the correct functioning of synapses [1]. The list of these synaptic proteins is growing rapidly, reflecting the diversity of synaptic complexes [2], including, neuroligins (NLs), SynCAMs, netrin-G, SALM3, SALM5, transmembrane proteins with leucine-rich repeats (LRRTMs), the slit − and TRK-like proteins (Slitrks) [1]. Calsyntenins belong to the cadherin superfamily and have binding sites with cadherins, α-laminin and neurexins [3]. Comparison of cadherin-11 to its mammalian and *Drosophila* homologues found that these proteins share significant similarity in their sequence along their whole lengths [4]. The mammalian homologues of cadherin-11 are termed calsyntenins because their cytoplasmic domains can bind synaptic calcium [5], thereby controlling its concentration, and furthermore binding to vesicles with kinesin-1 to predetermine intracellular transport [6]. The expression of calsyntenins is restricted exclusively in brain, with high levels occurring in cortical gamma-aminobutyric acid (GABA)ergic interneurons and in medial temporal lobe regions [7];hence, physiological functions of calsyntenins are related to the central nervous system and perhaps etiology of some psychiatric disorders [8].

There are three types of calsyntenins − 1, 2 and 3, which are expressed postsynaptically [7] and each of them has certain function in the brain. The targeted deletion of distinct calsyntenin genes disrupted synaptic functions in mice. Lack of calsyntenin1 (calsyntenin 1 knockout mice; Clstnt1-KO) led to neurodevelopmental delay of excitatory neurons and reduced branching of dendrites of the hippocampal neurons in Clstn1-KO mice [9]. The observed neuronal phenotypes appear to be related to Clstn1 protein interactions with light-

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chain kinesis-1 and related intracellular vesicular transport system [6]. Clstn3-KO mice have decreased density of excitatory and inhibitory neurons in the hippocampus which is related to Clstn3 protein interaction with the synaptic protein α–neurexin. This imlicates synaptogenesis as one of Clstn3's functions [10]. Calsyntenin-2 (γ-alcadein) plays important role in learning and memory. Calsyntenin-2 is associated with verbal memory in young humans, semantic memory and global cognitive abilities in elderly people [11]. The fMRI study showed effect of polymorphism in Clstn2 gene on functional neuronal connections between parahippocampal gyrus of the right hemisphere, bilateral caudal nuclei and frontal-cortical regions of brain during word recognition [12]. Polymorphism of the calsyntenin-2 gene was associated with modulation of early and delayed recall of words in a large sample of Swiss origin [13]. A recent study found that Clstn2 knockout (Clstn2-KO) mice have deficit of inhibitory GABAergic interneurons in the cortex and hippocampal CA1, CA3 areas, accompanied by hyperactivity and deficient hippocampus-dependent spatial learning & memory [14].

Overall, the functional role of calsyntenins in the brain is to regulate balance between excitatory and inhibitory neuronal functions. Notably, alterations in the ratio of excitatory to inhibitory cortical activity (E/I imbalance) may explain social and cognitive deficits, impaired emotional processing seen in patients with autism spectrum disorder (ASD) [15]. Indeed, postmortem studies detected structural and functional alteration in both glutamatergic and GABAergic inhibitory circuits in individuals with ASD [16]. Strikingly, a recent genetic study detected a deletion of the intron2 of Clstn2 gene, identified by analysis of copy number variance (CNV) in patients with ASD [17].

Though we already reported that calsyntenin-2 selectively regulates level of inhibitory synapses and spatial learning & memory in mice [14], *in vivo* studies focused on characterization of social and emotional phenotypes in Clstn2-KO mice are currently lacking. Here, using a battery of behavioral tests we identified social deficits exclusively in Clstn2-KO males, but not in females, without any changes in olfactory functions, excluding sensory deficit in knockout mice. Moreover, Clstn2-KO males, not females, demonstrated greater emotionality in response to highly aversive conditions. However, Clstn2-KO mice of both sexes showed hyperactivity and repetitive behavior. Altogether, our results demonstrated that lack of calsyntenin-2 models several features of ASD (social deficit, restricted interest), support a possible role of calsyntenin-2 in psychopathological mechanisms of ASD, although other behavioral endophenotypes of Clstn2-KO mice (hyperactivity, deficient spatial memory) are not directly related to this disorder.

2. Methods

2.1. Animals

Clstn2 homozygous knockout mice (Clstn2-KO) and WT mice were kept in the animal facility at the Institute of Physiology and Fundamental Medicine in standard cages (OptiMice Biotech A.S.; 34 × 29 × 15 cm) for 5 animals per cage. Animals were kept under inverted light regime (light from 18.00 p.m. to 6 a.m.) and temperature was maintained about 23 °C. Mice had *ad libitum* access to rodent food pellets (Ssniff, Germany) and water in home-cages. Generation of Clstn2-KO mice has been previously described [14]. To maintain this knockout line, Clstn2-KO mice were mated with C57BL/6NCrl mice. Next, Clstn2 heterozygous offspring were intercrossed to generate Clstn2-KO mice and their wild-type littermates (WT). Experimental animals were genotyped by PCR using primers: "cls2-ko-F1" (knockout forward: 5′ AAGTTTTGGGCTTGTAGATCCAGC TCT GTC) and "neo-ko-R1" (knockout reverse: 5′ AAATTGCATCGCATTGTCTGAGTA GGTGTC) and "cls20ko-R2" (wild-type reverse: 5′ GATGTCTTATTGAGCACCAC-AGCCTCAAAG). WT and Clstn2-KO amplicons were ~162 base pairs and ~364 base pairs, respectively. Homozygous Clstn2-KO mice and WT littermates from in house heterozygous breeding pairs were weaned at 3 weeks of age and tested at the age of 3–4 months. Female mice of both genotypes were at random (undetermined) stages of the estrus cycle. All animal procedures were conducted in agreement with the Russian Academy of Science based on European communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Behavioral experiments

Behavioral tests were done between 9 am and 4 pm on adult WT and Clstn2-KO mice. Prior to all experiments mice were acclimatized to the experimental room for 30 min. The behavioral equipment was cleaned with 70% ethanol between mice to remove residual odors. The subjective tests (e.g. open field, forced swim test, social affiliation\recognition, dyadic social test) were video-recorded and analyzed by skilled experimenter blind to genotype of the mice using EthoVision XT-10 software (Noldus Information Technology, Netherlands). Several cohorts of mice were used in the behavioral studies to minimize any influence of one behavioral procedure on another. The mice were given minimum 7-day interval between the tests.

2.2.1. Elevated plus-maze (EPM)

The elevated plus-maze is a traditional test for estimating the anxious state in rodents. The maze consists of two open arms (25 × 5 cm), two closed arms (25 × 5 × 30 cm), arranged so that the two arms of each type were opposite each other and extended from a central platform (5 × 5 cm). The floor and side-walls of maze were constructed from grey opaque Plexiglas material. The maze was elevated to a height of 50 cm. All measurements were taken in a dimly lit experimental room. The central platform of the EPM was illuminated by a lamp heighted for 1.5 m above the central platform. Each mouse was placed into the center of the EPM facing the closed arms. Over a 5-min test period, the following parameters were recorded: 1) open arm time, closed arm time and central platform time as a percentage of total testing time; 2) open arm entries, closed arm entries and central platform entries as a percentage of total entries; 3) total distance; 4) head-dips.

2.2.2. Open field (OF)

Each mouse was placed in the middle of activity cage (Plexiglas cage; 40cm × 40cm × 37 cm; regular room' light) for 5 or 30 min. The follow parameters were recorded: total travelled distance (for 5 and 30 min); time spent in the center (20 × 20 cm) and in the border (width of the marginal area was 10 cm) of the open field for 5 min. For brevity the ratio between time spent in the center and border is presented.

2.2.3. Marble burying test

This test was conducted in the standard cage of size (37 cm × 26 cm × 17 cm) under regular room light as previously described [18]. Each mouse was placed in the cage filled with 3 cm of bedding and allowed to explore 12 glass marbles arranged in 4 rows of 3 marbles equidistant from one another for 15 min. Afterwards, the mouse was removed and the number of buried marbles was recorded. A marble burying index was scored as 1 for marbles covered > 50% by bedding, 0.5 points − for half covered marbles, and 0 points − for less than half covered marbles.

2.2.4. Social affiliation and recognition

The experiment was performed as described [19] with slight modifications. In particular, two wired cylinders of the same size (diameter: 7.5 cm × height: 8 cm) were placed in the opposite corners among one side of the experimental Plexiglas chamber (40cm × 40cm × 37 cm). The procedure included three phases: *adaptation:* the studied mouse was placed in the center of the experimental chamber for 5 min and then returned into the home cage for 1–2 min. *Session 1*: "stranger 1" (unknown wild-type mouse of the same sex, age and weight) was placed

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