Archival Report

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CACNA1D De Novo Mutations in Autism Spectrum Disorders Activate Cav1.3 L-Type Calcium Channels

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ABSTRACT

BACKGROUND: Cav1.3 voltage-gated L-type calcium channels (LTCCs) are part of postsynaptic neuronal signaling networks. They play a key role in brain function, including fear memory and emotional and drug-taking behaviors. A whole-exome sequencing study identified a de novo mutation, p.A749G, in Cav1.3 α_1-subunits (*CACNA1D*), the second main LTCC in the brain, as 1 of 62 high risk–conferring mutations in a cohort of patients with autism and intellectual disability. We screened all published genetic information available from whole-exome sequencing studies and identified a second de novo *CACNA1D* mutation, p.G407R. Both mutations are present only in the probands and not in their unaffected parents or siblings.

METHODS: We functionally expressed both mutations in tsA-201 cells to study their functional consequences using whole-cell patch-clamp.

RESULTS: The mutations p.A749G and p.G407R caused dramatic changes in channel gating by shifting (~15 mV) the voltage dependence for steady-state activation and inactivation to more negative voltages (p.A749G) or by pronounced slowing of current inactivation during depolarizing stimuli (p.G407R). In both cases, these changes are compatible with a gain-of-function phenotype.

CONCLUSIONS: Our data, together with the discovery that Cav1.3 gain-of-function causes primary aldosteronism with seizures, neurologic abnormalities, and intellectual disability, suggest that Cav1.3 gain-of-function mutations confer a major part of the risk for autism in the two probands and may even cause the disease. Our findings have immediate clinical relevance because blockers of LTCCs are available for therapeutic attempts in affected individuals. Patients should also be explored for other symptoms likely resulting from Cav1.3 hyperactivity, in particular, primary aldosteronism.

Keywords: Autism spectrum disorders, Calcium channel blockers, Human genetics, L-type calcium channels, Neuropsychiatric disorders, Whole-exome sequencing

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L-type calcium channels (LTCCs; Cav1) are one of the three major classes (Cav1–Cav3) of voltage-gated calcium channels (1). They are expressed in most electrically excitable cells (1–3). Many body functions, including muscle contraction and brain, endocrine, and sensory functions, depend on proper LTCC activity (2–4). The LTCCs contain high-affinity drug-binding sites for different chemical classes of organic calcium channel blockers (5). Blocking of LTCCs in vascular smooth muscle and the heart has been therapeutically used for decades to treat elevated blood pressure and cardiac ischemias. The dihydropyridine class of LTCC blockers still belongs to the top-selling antihypertensives.

Despite their importance as peripheral drug targets, LTCCs play a key role for normal brain function. Within the LTCC family (Cav1.1–Cav1.4), Cav1.2 and Cav1.3 are the two isoforms expressed in the brain (3). They are located at postsynaptic somatodendritic sites, shape short-term and long-term adaptations of synaptic function (2,4,6,7), and are often present in the same neurons (6). However, despite high structural homology, they differ with respect to their gating properties and protein interaction partners (3). They contribute differently to various brain functions, such as emotional and drug-taking behaviors and different types of memory (2–4). Cav1.3 comprises only ~10% of the LTCCs in the brain (8), but because of its more negative activation voltage range, it can carry inward calcium currents at threshold voltages (9,10), shaping neuronal firing patterns and contributing to pacemaker currents (2,6,7,11).

Data from mouse studies and human channelopathies provide important insight into the potential role of Cav1.2 and Cav1.3 LTCCs in human brain disease. In genome-wide association studies and exome sequencing studies, *CACNA1C* has emerged as a new candidate gene for neuropsychiatric disease, including bipolar disorder, major depression,

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schizophrenia, and autism (12–15). Reduced Cav1.2 expression in the mouse forebrain results in anxiety-like behavior. Decreased channel function may contribute to the pathophysiology of anxiety in neuropsychiatric diseases (16). Timothy syndrome is a rare multiorgan disorder resulting from Cav1.2 gain-of-function mutations (OMIM No. 601005) (17), and surviving patients may also develop autism and epilepsy (17). Knock-in mice expressing the human mutation replicate autistic behavioral traits (18). Both gain and loss of Cav1.2 channel activity can lead to central nervous system dysfunction.

In contrast, heterozygous loss of Cav1.3 channel function does not result in a detectable phenotype in mice (3,19) and humans (20). Instead, the specific acute activation of Cav1.3 induces depression-like behaviors (8) and leads to activation of brain regions involved in anxiety and fear circuits (21). Gain-of-function of this channel may also underlie neuropsychiatric symptoms in humans. This possibility is further supported by the description of two patients with two different germline *CACNA1D* gain-of-function mutations (22,23). These mutations caused a severe congenital multiorgan syndrome with primary aldosteronism (22,23), seizures, and neurologic abnormalities (PASNA; OMIM No. 615474). Symptoms also included global developmental delay and intellectual disability (23) indicating that constitutively enhanced Cav1.3 activity interferes with normal neuronal function and development (22,23).

In the present study, we show that one *CACNA1D* mutation (p.A749G in Cav1.3 α1), which has been reported as 1 of 62 high risk–conferring mutations in a whole-exome sequencing (WES) study of patients with sporadic autism and intellectual disability (24), induces a strong increase in Cav1.3 channel function. We screened WES data in patients with sporadic autism for other *CACNA1D* de novo mutations and identified p.G407R in another patient, for which we also demonstrate a pronounced gain-of-function. Our data strongly support *CACNA1D* as a recurrent risk gene for autism spectrum disorder (ASD). Given the nature of the mutation, the severe congenital disorder in two other patients, and the pathophysiologic relationship to Timothy syndrome, our data strongly suggest possibly a causal role of *CACNA1D* gain-of-function mutations for ASD in these patients. This observation has immediate clinical relevance because clinically used blockers of LTCCs are available for immediate therapeutic intervention. Affected patients should also be monitored for other symptoms expected from Cav1.3 hyperactivity, in particular, primary hyperaldosteronism and hypertension (22,23).

METHODS AND MATERIALS

WES Data Analysis

Published studies reporting WES data from probands with sporadic autism were examined for de novo mutations in *CACNA1D*. Five studies (24–28) providing data on 980 probands were identified (Table S1 in Supplement 1). Two mutations, p.A749G (A749G; NM_000720 reference sequence) in proband 11872.p1 and p.G407R (G407R) in proband 12620.p1 in the Simons Simplex Collection, were reported in two separate studies (24,25). Both mutations were present in the patient only and not in family members. Both were not reported as variants in the Exome Variant Server (http://evs.gs.washington.edu), the Single Nucleotide Polymorphism Database, and the 1000 Genomes Project. Both were also confirmed as the only *CACNA1D* de novo mutations in a recent WES study including 2303 trios (which included the aforementioned cohort) (29).

Complementary DNA Constructs

The human wild-type Cav1.3 channel α_1-subunit (*CACNA1D* gene, National Center for Biotechnology Information reference sequence EU363339, long C-terminal splice variant) containing the alternative exons 8a and 42 was previously cloned into $pGFP^{minus}$ vector (mammalian expression plasmid controlled by cytomegalovirus promoter; it lacks a GFP tag) (30). The A749G and G407R mutations were introduced into the human Cav1.3 construct using standard polymerase chain reaction approaches. Mutated constructs were verified by DNA sequencing (Eurofins MWG Operon; Eurofins Genomics, Ebersberg, Germany).

Electrophysiologic Recordings in tsA-201 Cells

Cell culture and transient expression of Cav1.3 constructs in tsA-201 cells were performed as described elsewhere (31). Whole-cell patch-clamp recordings were performed at room temperature. Borosilicate glass electrodes were pulled (micropipette puller; Sutter Instrument Company, Novato, California) and fire polished (MF-830 Microforge; Narashige, London, United Kingdom) at a final resistance of 1.5–2.5 MΩ. Cells were recorded at a sampling rate of 2–5 kHz using an Axopatch 200B amplifier (Molecular Devices, Biberach, Germany), digitized with Digitizer 1322A (Molecular Devices), and recorded with pClamp 10.2 software (Molecular Devices). The recording solution contained in mmol/L: bath—15 calcium chloride, 10 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 150 choline chloride, and 1 magnesium chloride, adjusted to pH 7.4 with cesium hydroxide; intracellular—135 cesium chloride, 10 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, 10 cesium–ethylene glycol tetraacetic acid, 1 magnesium chloride, 4 mmol/L disodium adenosine 5′-triphosphate adjusted to pH 7.4 with cesium hydroxide.

Cells were held at a holding potential of −80 mV before a step protocol of 25 msec or 50 msec to different voltages was applied to determine the current-voltage relationship. Currents were leak subtracted using a P/4 protocol. The voltage dependence of inactivation was measured by applying a control test pulse (20 msec to the voltage of maximal inward current [V_{max}]) followed by 5-sec conditioning steps to various potentials and a subsequent 20-msec test pulse to V_{max} (30-sec recovery between protocols). Inactivation was calculated as the ratio between the current amplitudes of the test versus control pulse. Estimates for changes in channel open probability or single channel conductance were obtained as described previously (22) by normalizing the ionic tail current after a 20-msec or 25-msec depolarizing pulse to the reversal potential to the "on" gating current (Q_{ON}) obtained in the same pulse. Current-voltage curves were fitted to the equation $I = G_{max}(V - V_{rev})/\{1 + \exp[-(V - V_{.5})/k]\}$, where V_{rev} is the reversal potential, V is the test potential, I is the peak current, G_{max} is the maximum conductance, $V_{.5}$ is the half maximal