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The puzzling role of TRPC3 channels in Motor Coordination

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Abstract

Transient receptor potential canonical 3 (TRPC3) proteins are nonselective cation channels activated downstream of Phospholipase C (PLC)-coupled receptors. TRPC3 channels have emerged as major players in the function of the central nervous system (CNS). They have been described as important contributors to brain-derived neurotrophic factor (BDNF)-mediated survival and growth-cone guidance of cerebellar granule neurons. TRPC3 were also identified as postsynaptic cation channels essential for metabotropic glutamate receptor1 (mGluR1)-dependent synaptic transmission in cerebellar Purkinje neurons. A recent report described motor coordination defects in TRPC3 knockout mice while a subsequent study reported a similar phenotype in so-called moonwalker mice, harboring a TRPC3 gain of function mutation. How can opposing aspects of TRPC3 channel activation lead to the same phenotype? Here we discuss the salient features of TRPC3 knockout mice and moonwalker mice and attempt to reconcile the apparently conflicting findings from these two animal models.

Introduction

The mammalian TRPC3 protein belongs to the transient receptor potential canonical (TRPC) family of nonselective cation channels which is part of the larger TRP superfamily(23, 30, 33). TRPC channels are the closest mammalian homologs to *drosophilae trp* and are activated through Phospholipase C (PLC)-coupled receptors(30). TRPC channel family can be subdivided into 3 major subfamilies: TRPC1/4/5; TRPC2 (a pseudogene in humans but encodes a functional protein in rodents); and TRPC3/6/7(33). It is clearly established that TRPC3/6/7 subfamily members are activated by diacylglycerol (DAG), produced downstream of Phospholipase C (PLC)-coupled receptors, in a protein kinase C (PKC)-independent manner(12, 24, 31, 32). In addition to its role in generating DAG, the phosphatidylinositol 4,5-bisphosphate (PIP₂) appears to be required in the mechanism of DAG activation of TRPC3/6/7 channels(19). Interestingly, the activation of these three channels by DAG analogs is inhibited by phorbol-12-myristate-13-acetate (PMA)(24, 31, 35), suggesting a role for PKC-mediated phosphorylation in channel inhibition. A serine residue at position 712 (S712) in human TRPC3 has been identified as the major site mediating PKC phosphorylation(29). PKG-mediated phosphorylation of TRPC3 on threonine11 (T11) and serine 263 (S263) was also shown to suppress channel activity(17). The PKC-mediated inhibition of TRPC3 channel function was proposed to occur directly and indirectly through PKG-mediated phosphorylation(18) (Figure 1). Several reports have proposed a role of TRPC3 channels in encoding components of the store-operated Ca²⁺ entry pathway, triggered by the depletion of internal Ca²⁺ stores (reviewed in(30)).

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Conflict of interest

None to declare.

However, other investigators refuted such participation and the role of TRPC channels in general in store-operated Ca^{2+} entry remains a highly contentious issue.

TRPC3 and neuronal function

TRPC3 proteins form nonselective cation channels that are ubiquitously expressed in both excitable and non-excitable tissues but their expression is predominant in specific regions of the brain and heart(7, 10). TRPC3 proteins were detected in rat brain during embryonic development and in the adult cerebral cortex; TRPC3 was shown highly enriched in the cerebellum(13). The exact mechanisms connecting TRPC3 to downstream functional inputs and how TRPC3-dependent Ca^{2+} or Na^{+} influx mediate TRPC3 function, remain incompletely understood. It has been suggested that some of the actions of TRPC3 in excitable cells are critically dependent on functional associations with other transport systems such as voltage-gated Ca^{2+} channels and/or $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers (NCX)(7). According to this view, Na^{+} entry through TRPC3 channels might either couple to Ca^{2+} uptake through NCX (7–9, 26) or provide the depolarization needed for activation of voltage-gated Ca^{2+} channels, as previously reported for TRPC6 in smooth muscle cells(27) (Figure 1).

The role of TRPC3 channels in the central nervous system (CNS) is starting to emerge (for a recent review see: (28)). The brain-derived neurotrophic factor (BDNF), an important modulator of cell proliferation, differentiation and survival, transmitter release and activity-dependent synaptic plasticity in the central nervous system (CNS), was shown to activate a nonselective cation current encoded by TRPC3 in pontine neurons(20). BDNF was shown to mediate guidance of nerve growth cones in rat cerebellar granule neurons through activation of TRPC3-mediated Ca^{2+} entry(21). Amaral and Pozzo-Miller reported the activation by BDNF of slowly developing and sustained nonselective cationic, membrane-depolarizing currents in CA1 pyramidal neurons that were absent in cells treated with small interfering RNA against TRPC3(1). In these cells, TRPC3 channels were shown to be necessary for BDNF-mediated increase in dendritic spine density. An interesting finding in this study is that the sustained BDNF-activated current was dependent on phosphatidylinositol 3-kinase-mediated TRPC3 insertion in the plasma membrane, as demonstrated by surface biotinylation(1). In cerebellar granule neurons, TRPC3 channels, along with TRPC6 were shown to promote survival. TRPC3 and TRPC6 protected against serum deprivation-induced apoptosis(14). Knockdown of TRPC3 or TRPC6 abrogated BDNF-mediated survival, BDNF-triggered intracellular Ca^{2+} elevation and BDNF-induced CREB activation, while overexpression of TRPC3 or TRPC6 increased CREB activation and prevented apoptosis in granule neurons deprived of serum(14). Furthermore, *In vivo* knockdown of TRPC3 or TRPC6 in neonatal rat cerebellums induced apoptosis of granule neurons which was reversed by overexpression of either TRPC3 or TRPC6(14). Data from Berg et al suggested that metabotropic glutamate receptors 1 and 5 (mGluR1/5) mediate glutamatergic excitation of striatal cholinergic interneurons mainly through the activation of TRPC3/TRPC7-like cationic currents; the expression of TRPC6 was not detected in these cells(3).

An important feature of TRPC3 channels that distinguishes them from close homologs such as TRPC6 channels is their substantial constitutive activity(4). TRPC3 is monoglycosylated while TRPC6 contains 2 glycosylation sites and introduction of a second glycosylation site in TRPC3 yielded a channel with markedly reduced constitutive activity(5). This constitutive activity of TRPC3 might be important in driving specific cell functions in the absence of active stimuli. As previously suggested by Groschner and colleagues(7), increased expression of TRPC3 at the plasma membrane may induce significant changes in excitable cells by causing membrane depolarization. For instance, the compensatory upregulation of TRPC3 channels in vascular smooth muscle of TRPC6 knockout mice was

responsible for the unexpected increase in vascular reactivity in these animals(6). Similarly, pulmonary vascular medial hypertrophy in patients with idiopathic pulmonary hypertension correlated with increased TRPC3 expression in pulmonary artery smooth muscle(34). In CNS, BDNF-mediated TRPC3 insertion in the plasma membrane of CA1 pyramidal neurons described by Amaral and Pozzo-Miller(1), might play an essential role in regulating the long term actions of BDNF. A recent study reported an important role for the constitutive activity of TRPC3 channels in regulating basal ganglia output neurons. These cells are GABA projection neurons of the substantia nigra pars reticulata (SNr GABA) that are crucial for movement control and characterized by depolarized membrane potential and rapid spontaneous spiking(37). The pharmacological inhibition of TRPC3 by Flufemic acid or by an anti-TRPC3 antibody causes membrane hyperpolarization, reduction in firing frequency and enhanced firing irregularity in SNr GABA projection neurons(37). A follow up study from the same group suggested that dopamine dendritically released from dopamine neurons of the substantia nigra pars compacta (SNc), tonically excites SNr GABA neurons *via* dopamine 1 and 5 receptor co-activation causing further enhancement of TRPC3 constitutive activity(36). This mechanism is proposed to form an ultra-short “SNc to SNr” dopamine pathway, as an alternative to the established long-distance nigro-striato-nigral loop, that would regulate the firing intensity and pattern of these basal ganglia output neurons(36). The above mentioned studies highlight the important role played by TRPC3 channel constitutive and receptor-regulated activities in CNS function.

TRPC3 and motor coordination

The term “cerebellar ataxia” refers to a large group of neurological conditions characterized by defects in motor coordination due to dysfunction of the cerebellum. Cerebellar ataxias are either sporadic or acquired with wide spectrum etiologies including but not limited to environmental, endocrine, infectious and genetic causes(22). Two recent reports have focused on the role for TRPC3 channels in motor coordination in mice(2, 11). These reports described apparently similar motor coordination defects in TRPC3 knockout mice(11) and in mice with a TRPC3 gain-of-function mutation(2), and thus present a fundamental paradox.

On one hand, using knockout mice Hartmann et al have provided evidence for a role of TRPC3 channels in the slow synaptic excitation mediated by mGluRs in cerebellar Purkinje neurons(11). These authors generated TRPC3 knockout mice by excising exon 7 from the TRPC3 gene using a Cre-loxP-based strategy; exon 7 encodes the pore region and sixth transmembrane domain of TRPC3 channels (Figure 2). The disrupted TRPC3 gene in TRPC3 knockout mice is expected to encode a shorter protein of 606 amino acids lacking the C-terminal part downstream of the pore region(11). However, it is unclear whether this truncated TRPC3 protein is actually expressed in the TRPC3 knockout animals described by Hartmann et al(11). TRPC3 knockout mice lacked both slow synaptic potentials and mGluRs-activated inward currents, and exhibited an impaired walking behavior that was not as severe as ataxic phenotypes such as those observed in mGluR-deficient mice(11). These mice looked otherwise normal in size and appearance; the size and the layering structure of the cerebellum as well as the morphology of their Purkinje neurons including dendritic arbors were normal(11). Remarkably, TRPC1/4/6 triple knockout mice used in the same study displayed normal mGluRs-mediated synaptic transmission, strongly indicating functional specificity of TRPC3 in mGluRs-mediated signaling(11); however, see an earlier study by Kim et al which reported the activation of TRPC1 by mGluR1 in cultured rat cerebellar slices(16). In another TRPC3 knockout model, in which TRPC3 transcripts are absent due to the disruption of the promoter region of TRPC3, a severe phenotype has been described where mice developed atrophy and progressive paralysis from the caudal to the cephalic region accompanied by diffuse astrocytosis. These TRPC3 knockout mice die within 3–4 months(25).

On the other hand, Becker et al identified a heterozygous TRPC3 point mutation, in the so called *moonwalker* mouse (Mwk/+), where Threonine 635 is mutated to Alanine (T635A) and proposed that T635A mutation in TRPC3 is responsible for the Mwk/+ mice phenotype (Figure 2). These animals display abnormal Purkinje cell development and cerebellar ataxia(2). Becker et al proposed that T635 in mouse TRPC3 is phosphorylated by PKC γ . The TRPC3 T635A mutant is thus proposed to yield a channel with altered gating and increased sensitivity to lower concentrations of mGluRs agonists, causing progressive loss of Purkinje neurons by apoptosis and cerebellar ataxia. The evidence supporting this was based only on an *in vitro* kinase assay where the 29 amino acid S4/S5 linker of TRPC3 containing either T635 or the T635A mutation as a GST fusion was differentially phosphorylated by purified PKC γ . However, without evidence for the equivalent *in vivo* phosphorylation, the role of PKC in phosphorylating T635 on mouse TRPC3 remains uncertain.

Previously, using mutagenesis and *in vivo* phosphorylation assays on full length TRPC3, we clearly demonstrated that a highly conserved Serine residue at position 712 (S712) in human TRPC3 was necessary and sufficient for PKC-dependent regulation of the channel. TRPC3 channels in which S712 was the only site mutated to alanine (S712A) failed to show any phosphorylation *in vivo* or inhibition of their function upon addition of PMA(29). An alternative mutation of T573 to Alanine (T573A), that corresponds to mouse T635A mutation, prevented TRPC3 expression in HEK293 cells (Figure 2) (29). Subsequent studies by Kwan et al confirmed the role of S712 in PKC-mediated phosphorylation of human TRPC3(18). Another group independently demonstrated that the equivalent residues of S712 on isoforms A and B of rat TRPC6 are phosphorylated by PKC in PC12D cells(15). Hence, S712 is the critical site for PKC-mediated phosphorylation of TRPC3 channels *in vivo*. Becker et al showed that the mutant T635A of green fluorescent protein (GFP)-tagged TRPC3 could be visualized when transfected into COS cells. However, whether the T635A mutant protein is actually expressed in Mwk/+ mice is less certain. Hartmann et al showed that TRPC3 knockout mice display defects of motor coordination not as severe as ataxic animals(11). Intuitively, if Mwk/+ mice have only half of their functional TRPC3 channels, contributed by the wild type allele, they would be expected to have a less severe phenotype than that of TRPC3 knockout mice, which is not the case. Moreover, the increased sensitivity of Purkinje neurons from Mwk/+ mice to the mGluRs agonist dihydroxyphenylglycine (DHPG) argue against this possibility(2). A simpler explanation for the increased sensitivity to DHPG in Purkinje cells from Mwk/+ mice might be the result of more TRPC3 channels in plasma membranes of Purkinje neurons from Mwk/+ mice compared to wild type mice. In the Western blot data presented by Becker et al, Mwk/+ mice seem to express more TRPC3 protein than the wild type mice(2). Increased constitutively active TRPC3 channels at the plasma membrane of Purkinje neurons might contribute to aberrant signaling accounting for the Mwk/+ ataxic phenotype. Furthermore, the ectopic expression of TRPC3 T635A mutant in COS cells is unlikely to recapitulate the *in vivo* condition in mice. Ectopic expression in COS cells would likely generate mostly homotetramers of the mutant protein. However, the stoichiometry in Purkinje cells from Mwk/+ mice is more complex, with heterotetramers contributed by any number of mutant and wild type TRPC3 molecules. Heteromultimerization with other TRPC isoforms seems unlikely in light of the normal mGluRs-mediated synaptic transmission in TRPC1/4/6 triple knockout mice(11). Ideally, *in vivo* phosphorylation should be assayed on TRPC3 proteins from cerebellum of Mwk/+ compared to cerebellar TRPC3 from wild type mice in order to support the view that T635 is phosphorylated.

The study by Hartmann et al provides a logical explanation of how lack of functional TRPC3 currents that are necessary for the slow synaptic excitation through mGluRs in Purkinje neurons could lead to a form of motor dysfunction. However it is less clear how

altered TRPC3 channel gating in Mwk/+ could lead to ataxia. Becker et al proposed diminished dendritic arborization and increased apoptosis in Purkinje neurons as potential mechanisms(2). While effects on Purkinje cell dendritic arborization and gait abnormalities in Mwk/+ mice were observed at 3 weeks of age, cell apoptosis only started to emerge in mice with 4 months of age and became apparent at 6 months. Huang et al showed significant upregulation of TRPC3 in rat cerebellum during the first 6 weeks after birth with the biggest increase in TRPC3 expression occurring between P1 and P16; TRPC3 expression being highly enriched in Purkinje neurons and their dendrites(13). Thus, the reason why Purkinje cells from Mwk/+ mice do not undergo apoptosis earlier is puzzling. Conceptually, other cell types expressing TRPC3 in Mwk/+ mice are potential targets for death by apoptosis and might contribute to the Mwk/+ mice ataxic phenotype.

Concluding remarks

A comparative summary of the main features of TRPC3 knockout mice and Mwk/+ mice is depicted in Table 1. Clearly, additional work is needed to understand the various components of the signaling cascade initiated by TRPC3 in the CNS and the differential connection of this cascade to specific functional outputs in different cell types. In light of recent findings implicating TRPC3 in BDNF-mediated survival of granule neurons(14), Becker et al, analyzed the cell death of granule neurons from wild-type and Mwk/+ mice upon growth factor deprivation and BDNF stimulation and found no significant difference in survival between wild-type and Mwk/+ granule neurons(2). How does signaling downstream of T635A TRPC3 specifically affects the survival of Purkinje neurons without affecting that of granule neurons is an important question that remain to be answered.

TRPC3 knockout mice described by Hartmann et al, presumably expressing a truncated TRPC3 protein, are normal in size and appearance(11). What would be the phenotype of Purkinje neurons if TRPC3 protein expression was totally absent? Does the truncated TRPC3 protein, encoded by the disrupted TRPC3 gene in knockout mice, express normally at the plasma membrane and fulfill some scaffolding or conformational role in cell signaling despite its inability to conduct cations?

Mwk/+ mice are of smaller size compared to their wild-type littermates, and the homozygous mutants (Mwk/Mwk) are not viable(2). At what stage of development does the homozygous T635A mutation in TRPC3 cause death and how? How does signaling downstream of T635A TRPC3 specifically affects the survival of Purkinje neurons? What are the downstream signaling pathways initiated by T635A TRPC3 in cells? The answers to these questions and others are required to resolve the apparent paradox between the results of Hartmann et al(11) and Becker et al(2). Further work in this direction is likely to enhance our understanding of the various modes of regulation and activation of TRPC3 and perhaps lead to novel pharmacological tools for treatment of cerebellar ataxia and other neurological disorders in which TRPC3 may play a role. The studies by Hartmann, Becker and their colleagues represent important first steps towards this goal.

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Figure 1. Activation mechanisms of TRPC3 Channels

Agonists such as BDNF and glutamate, by binding to specific receptors coupled to isoforms of Phospholipase C (PLC γ and PLC β respectively), mediate the generation of second messengers inositol 1,4,5 trisphosphate (IP $_3$) and diacylglycerol (DAG) through PLC-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP $_2$). Subsequently, Ca $^{2+}$ and Na $^{+}$ entry through nonselective TRPC3 channels is activated by DAG in a yet to be defined mechanism. PIP $_2$ is required for the activation of TRPC3 channels by DAG. Protein kinase C (PKC) exerts an inhibitory effect on TRPC3 channel activity directly through phosphorylation or indirectly *via* protein kinase G (PKG)-mediated phosphorylation. Ca $^{2+}$ entry through TRPC3 channels can activate downstream signaling pathways that control various cellular functions such as cell survival and synaptic transmission. An indirect coupling between TRPC3 and other transport systems have been proposed in other cell types whereby Na $^{+}$ entry through TRPC3 channels provides the depolarization necessary to activate voltage-operated Ca $^{2+}$ channels (VOC). Na $^{+}$ entry through TRPC3 channels was also proposed to couple to the Na $^{+}$ /Ca $^{2+}$ exchanger (NCX) functioning in its reverse mode and allowing Ca $^{2+}$ uptake into the cytoplasm. TRPC3 channels are known to display significant constitutive activity. The control of cell functions that are contingent on TRPC3 constitutive activity could be achieved by increased *de novo* insertion of TRPC3 proteins at the plasma membrane.

Figure 2. Structure of TRPC3 channels at the plasma membrane

(A) TRPC3 have cytoplasmic N- and C-termini, six transmembrane regions (TM; orange), a pore region (blue), a hydrophobic segment which does not seem to span the plasma membrane (gray), and four ankyrin-like repeats in the cytoplasmic N-terminal region (shown green in panel B). Locations of different residues shown to be phosphorylated by PKC (Serine 712; S712) and PKG (Threonine 11; T11 and Serine 263; S263) on human TRPC3 are shown. Threonine 573 (T573) is the human equivalent of Threonine 635 (T635) in mouse TRPC3. T635 which is mutated to Alanine in Mwk/+ mice is located in the cytoplasmic loop region between TM4 and TM5.

(B) Wild-type mouse TRPC3 (wt-mTRPC3) contains 836 amino acid. In TRPC3 knockout mice described by Hartmann et al, exon 7 is excised (corresponding to amino acids 601-666). A stop codon introduced after the first 5 amino acids encoded by exon 8 would generate a truncated TRPC3 protein with 606 amino acids (KO-mTRPC3); it is unclear whether this truncated protein is expressed in the knockout mice.

Table 1

Comparison between TRPC3 KO mice and Mwk/+ mice.

TRPC3 KO mice	Mwk/+ mice
Truncated protein of 606 amino acid, lacking pore and downstream C- terminus	Point mutation in TRPC3 gene yielding a protein in which threonine 635 is replaced by alanine (T635A)
Homozygote	Heterozygote (Mwk ^{-/-} homozygote is lethal)
Mouse size and general appearance normal	Mwk/+ are ~60% the size of their wild- type counterparts
Size and layering structure of cerebellum normal	General size and structure of cerebellum normal
Morphology of Purkinje neurons and their dendrites normal	Reduced dendritic arborization of Purkinje neurons in 3 week old Mwk/+ mice; at 4 months of age, Mwk/+ mice showed a slow and progressive loss of Purkinje neurons by apoptosis; at six months, extensive loss of Purkinje neurons, particularly in the lateral cerebellar hemispheres was noted; no change in observed cerebellar granule neurons
Fast synaptic transmission at parallel fiber synapse normal	Not reported
Presynaptic glutamate release normal	Not reported
Calcium release from intracellular stores normal	Not reported
mGluR1-mediated slow excitatory postsynaptic potential (EPSP) abolished	Increased sensitivity to DHPG. At high DHPG concentration (20μM), mGluR-mediated inward current in Purkinje neurons showed smaller spikes and a faster interspike interval compared with wild type animals; at low concentration of DHPG (5μM), clear inward current in Mwk/+ Purkinje neurons but undetectable currents in wild type
Deficient motor coordination; mice walked in a straight line but hindpaws footprints outside width of the transverse body diameter; poor performance walking on irregularly spaced ladder and thin elevated beam compared with wild type mice	Ataxia; severe gait abnormalities including retropulsion from 3 weeks of age; meandering walk and significantly broader gait width; non-uniform alternating left-right step pattern; impaired balance and walk across a beam