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Research:

Neuronal Mechanisms of Spino-Cerebellar Ataxia Type 27

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Abstract
Spinocerebellar ataxia type 27 (SCA27) is an autosomal dominant genetic disorder due to loss of function mutations of the FGF14 gene. In addition to ataxia, patients with SCA27 also present with intellectual disability and, sometimes, paroxysmal dyskinesia. The consequences of deficiency of the FGF14 protein have been studied in Fgf14 knock-out mice, which recapitulate the symptoms of SCA27. Fgf14 plays a pivotal role in the localization of voltage-dependent sodium channels at the initial segment of axons, determining the level of neuronal excitability and the generation of action potentials. The interaction of Fgf14 with voltage-dependent sodium channels is finely controlled by several transduction pathways via kinases including GSK3, CK2 and Wee1. The excitability of neurons expressing Fgf14 is profoundly altered by its deletion, so that action potential firing is suppressed in hippocampal pyramidal neurons and in cerebellar granule and Purkinje cells. The ataxic symptoms can be attributed to the lack of Fgf14 in the cerebellar cortex, as shown by Fgf14 knock-down experiments. The paroxysmal dyskinesia might be related to functional alterations of basal ganglia, also related to blunted responses to dopamine agonists and to drugs of abuse like cocaine and amphetamine. Some cognitive symptoms of SCA27 patients and Fgf14 knock-out mice, like deficits in working and long-term memory, have been attributed to functional alterations of the hippocampus, including a decrease of inhibitory signaling from fast spiking inter neurons and incomplete maturation of neurons born by adult neurogenesis in the dentate gyrus. In addition, Fgf14 is required for proper neurotransmitter release and synaptic transmission in cerebellum and hippocampus. FGF14 is an emerging pivotal element in the fine-tuning of complex cerebral networks implied in psychiatric disorders.

Keywords: Fibroblast growth factors; FGF14; Ataxia; SCA27; Dyskinesia; Cerebellum; Hippocampus; Axon initial segment; Neuronal excitability; Psychiatric disorders

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Introduction

Genetic and clinical features of SCA27

Spinocerebellar ataxias (SCAs) are a heterogeneous group of inherited neurodegenerative diseases affecting the cerebellum with different degrees of involvement of the brainstem and sometimes also of other brain regions (for review see ref. [1]). They have an autosomal dominant pattern of transmission. Clinically, they are characterized by progressive cerebellar ataxia, often associated with pyramidal or extrapyramidal signs, ophthalmoplegia, cognitive impairment. Extra pyramidal signs include parkinsonism, early onset tremor, epilepsy.

The SCA type 27 (SCA27) was first described by van Swieten et al., [2] in a family with slowly progressing early-onset tremor, dyskinesia and slowly progressive cerebellar ataxia. These patients also displayed low IQ and deficits in memory and executive functioning [3]. Affected individuals bear a mutation on chromosome 13q34 in the gene fibroblast growth factor 14 (FGF14) also known as fibroblast growth factor homologous factor 4 (FHF4) [4]. The FGF14 gene contains 5 exons, with two alternative-splicing variants of exon 1 [5]. Two major mRNA transcripts are produced, FGF14a and FGF14b, which differ in the definition of exon 1 [6]. The two transcripts encode protein isoforms of 247 and 252 amino acids, respectively. A missense T > C mutation was identified at position 434 of the FGF14 ORF in exon 4, resulting in the substitution of a serine for a phenylalanine at position 145 (F145S). F145 is in a highly conserved region, probably located in the hydrophobic core of the protein. Modeling the structure of the FGF14 protein led to the conclusion that the F145S mutation acts indirectly, by reducing its stability. For this reason, it has been hypothesized that the mutation might have a dominant-negative effect on the FGF14 protein [2]. Subsequent studies described FGF14 mutations, which were more in line with a haploinsufficiency mechanism. Dalski et al., [7] identified a patient with ataxia and mental retardation, heterozygous for a frameshift mutation of FGF14 leading to a premature truncation of the protein (p.Asp163fsX12). In 2009 Misceo et al., reported a chromosomal translocation with the breakpoint between exons 1 and 2 of the b isoform of FGF14 in patients with signs of cerebellar dysfunction, extrapyramidal tremors, dyskinesia, peripheral neuropathy, impairment of positional sensation, and mental retardation [8]. Ataxia and tremor had a very early onset as they were evident already at the age of 2 years. This report is in agreement with the FGF14 loss of function hypothesis of SCA27, in this case pointing to haploinsufficiency rather than dominant negative effect. A similar mechanism, a translocation disrupting FGF14, has been described by Shimojima et al., [9] in a patient diagnosed with paroxysmal non-kinesigenic dykinesia (PNKD). Also in this case the mechanism can be attributed to haploinsufficiency.

The exon 4 of FGF14 was screened in a study of an Asian population, but no mutation was found in 105 patients with either ataxia or postural tremor [10]. Another study conducted in Asia found a deletion variation (c.-10delC) in the 5’UTR region of FGF14 exon 1b in a Chinese family, while in other 67 patients with ataxia no FGF14 mutation was present [11]. Taken together, these two studies indicate that SCA27 is a rare cause of ataxia in Asian populations.

Interestingly, Coebergh et al., [12] reported a case, with early onset at two years of age and with ataxic symptoms that were aggravated by fever. The patient had a deletion encompassing part of FGF14. The mother and grandmother of the proband had the same deletion and clinically they displayed an episodic ataxia with nystagmus and postural unbalance.
Aggravation by fever is in line with the role of FGF14 on voltage-dependent sodium channels (NaV), whose function is strongly temperature-dependent. For example, mutations of SCN1A, coding for the NaV1.1 channel, are responsible for a genetic epilepsy characterized by febrile seizures [13,14]. An ataxia with an episodic pattern of expression was also found by Choquet et al., [15] in a family with an FGF14 mutation causing a frameshift and a premature stop codon. A very early onset case, with axial ataxia and intention tremor of the upper limbs extremities already noted at age 1 year, has been described by Planes et al., [16]. Genetic analysis revealed a deletion involving at least the first 2 exons of FGF14. These clinical studies are in accordance in pointing to loss of function of FGF14 with haploinsufficiency as the mechanism of autosomal dominant SCA27.

**Animal models of SCA27**

In order to generate mice with a deletion of the Fgf14 gene (Fgf14-KO), Wang et al., [5] replaced the second and third exon with the β-galactosidase gene (β-gal). While heterozygous mice were indistinguishable from their wild-type littermates, homozygotes were viable and fertile but displayed several neurologic symptoms. Fgf14-KO mice showed ataxia and paroxysmal dyskinesia starting at 3 weeks of age. The body weight of Fgf14-KO mice was indistinguishable from wild-type until weaning, but in the adulthood remained about 15% lower [5]. By detection of β-gal in Fgf14-KO mice, the expression of Fgf14 transcripts was revealed, with a strong staining in the granule cell layer of the cerebellum, in hippocampus, parahippocampal region, striatum, amygdala [5,17]. β-gal RNA blot analysis showed that the highest levels of Fgf14 expression were attained in the cerebellum, followed by hippocampus, amygdala, cerebral cortex, striatum and thalamus. The protein localization was detected in the molecular layer of the cerebellar cortex, with a pattern typical of granule cell parallel fibers. In this structure and in the striatum the expression of Fgf14 was strong, while a moderate expression was found in cerebral cortex, hippocampus, hypothalamus, amygdala [5,17]. The corpus callosum was also intensely stained, confirming that the Fgf14 protein is highly localized in the axons of specific types of neuron, like granule cells of the cerebellum and pyramidal neurons of the cerebral cortex [5]. In Fgf14-KO mice, the brain structure was intact, with normal organization of cell bodies and absence of signs of demyelination [5].

With an analysis of motor behavior, Fgf14-KO mice showed a variable and irregular gait with shorter stride and lower forepaw-hindpaw correspondence [5,18]. These features are reminiscent of ataxic gait. In fact, Fgf14-KO mice could remain on a rotating rod only a few seconds and their performance failed to improve with training [5,18]. The role of Fgf14 in motor control has been confirmed in a different murine model, in which Fgf14 expression was knocked-down by shRNA [19]. In this study, the expression of Fgf14 was acutely and selectively suppressed by stereotaxic injection in the PC layer of adeno-associated virus containing shRNA targeted to Fgf14. Four weeks after the injection the mice showed a clear deficit in the balance beam test. This result confirms that at least part of the motor impairment is due to dysfunction of the cerebellar cortex.

Fgf14-KO mice, in which Fgf14 is constitutively deleted in all brain regions, at the age of about 1 month developed episodes of clonic spasms of the forelimbs and hyperextension of the hindlimbs, with loss of balance and tremor [5]. Such episodes lasted about 10 minutes and occurred several times per day [5]. It is interesting to note that, instead of an aggravation with age, at more than 3 months these symptoms became less severe [5].

Electroencephalography during these episodes ruled out the hypothesis of epileptic seizures, so that they were classified as paroxysmal dyskinesia [5]. Paroxysmal dyskinesia is a rare motor disorder with dystonic and choreiform movements [20]. It is important to recall that paroxysmal non-kinesigenic dyskinesia was found in a patient with a disruption of FGF14 [9]. It is interesting that these dyskinetic episodes are very similar to episodic ataxia (EA) and patients with EA type 1 can also have paroxysmal kinesigenic dyskinesia [20].

In addition to the ataxic and dyskinetic symptoms, the administration of an epileptic form drug uncovered an increased susceptibility to seizures [5]. This aspect has not been further investigated.

**Fgf14 and Control of Neuronal Excitability at the Axon Initial Segment**

**Functional roles of Fgf14 at the AIS**

The axonal initial segment (AIS) is a highly specialized structure critical for initiation of the action potential in neurons (for review see ref. [21]). The AIS is enriched in scaffolding and regulatory proteins that interact with ion channels such as Na and potassium channels controlling neuronal excitability [22-24]. Several studies confirmed the presence of Fgf14 at AIS of many types of neurons, as firstly reported by Lou et al., [25], and its role in the regulation of different Na-channel isoforms. Fgf14 interacts directly with the C-terminus of Naα subunits, including Na1.1, Na1.2, Na1.6 in HEK293 cells [25-28]. Similarly to the phenotypic consequences resulting from loss of function mutations in Na1.1 and Na1.6 [29-31], the deletion of Fgf14 remarkably attenuated excitability of cerebellar Purkinje cells [PCs] and granule neurons [18,32,33] and of hippocampal neurons [26,27,34]. Fgf14 co-localizes with Na channels in primary cultures of hippocampal neurons [23-27,35,36], in the AIS of cerebellar granule cells [37], in hippocampal and para-hippocampal regions [34], in the cortex and the NAc [38]. Xiao et al., [24] demonstrated that Fgf14 immunoreactivity is high in the AIS of PCs with a decreasing gradient distribution from proximal to distal segment. This pattern is evident early in the postnatal development of PCs and it has also been observed in many other types of central neurons such as mature hippocampal and cortical neurons. PCs express Na1.1 and Na1.6, and mice lacking either Na1.1 or Na1.6 show defects in firing, suggesting that both Naα subunits are required for normal PC function [29,39-40]. The distribution of Fgf14 and Na1.1 appears to be coordinately regulated. In the AIS of PCs in mice lacking Na1.6 there were increased levels of Fgf14 and Na1.1, and in the absence of Fgf14 all AIS Naα subunit levels in PCs were reduced. The preferential distribution of Na1.1 channels in the proximal part of AIS was lost and weak presence of Na1.1 was evident distally suggesting expansion of the Na1.1 along the AIS of Fgf14−/−PCs. These findings suggested a role for Fgf14 in regulating the densities and distributions of Na-channels in the AIS of mature PCs [24] and of hippocampal neurons, in which overexpression of Fgf14 resulted in increased levels of Naα subunits and increased Na-current densities [25-26]. The absence of Fgf14 in PCs did not appear to affect the distribution patterns of the AIS scaffolding proteins ankyrin G and βIV-spectrin [24], suggesting that Fgf14 is not required to initiate or maintain the structural components of the AIS. Fgf14 may function to regulate the affinity of Naα subunits to scaffolding proteins in the AIS or to other intermediary proteins that could couple Naα subunits to the protein components of the AIS.
scaffold such as ankyrin G and βIV-spectrin, and the extracellular cell adhesion molecules neurofascin-186 and neuron glia-related cell adhesion molecules [41-42]. Chronic depolarization of dissociated hippocampal neurons caused a relocation of the components of the AIS, including Na+, Fgf14, β-IV-spectrin and neurofascin, distally from the soma. This effect was associated to an increase in the current threshold for firing an action potential [35]. Recently Pablo et al., [36] reported that in hippocampal neurons Fgf14 binds directly to Na channels to promote their axonal localization while the homologous Fgf13, implicated in excitability control and genetic epilepsy [43], limits their somatodendritic surface expression by selective somatodendritic endocytosis. Deficits in excitability induced by interference with the molecular components and location of the AIS protein network could be relevant for the onset of a variety of psychiatric and neurological disorders [44-45].

Laezza et al., [27] showed that the subcellular targeting of Fgf14 to AIS of hippocampal neurons requires the N-terminus of the molecule and 2 N-terminal alternatively spliced Fgf14 variants, Fgf14-1a and Fgf14-1b, differentially regulate currents produced by Na1.2 and Na1.6 channels, suggesting that Fgf14 alternative splicing could represent one of the mechanisms involved in functional regulation of Na-channels.

Fgf14 binds directly to the Na1.6 channel C-tail, regulating channel gating and expression, and the residues Lys-74/Ile-76 at the N-terminal of Fgf14 have a key role in the Fgf14-Na1.6 complex and Fgf14-Fgf14 dimer formation as reported by Ali et al.,[28]. In addition, an alanine (Ala) mutation of Val-160 in the β-9 sheet of Fgf14 impaired binding to Na1.6 but had no effect on Fgf14-Fgf14 dimer formation. They demonstrated that either the Fgf14(V160A) or the Fgf14(K74A/I76A) mutation was sufficient to abolish the Fgf14-dependent regulation of peak transient Na+ currents and the voltage-dependent activation and steady-state inactivation of Na1.6; but only V160A with a concomitant alanine mutation at Tyr-158 could prevent Fgf14-dependent modulation of the channel fast inactivation. The identified amino acid residues with critical roles at the protein-protein interaction interface of Fgf14- Na1.6 may represent novel drug targets for modulating Na-channel activity and neuronal excitability.

**Modulation of Fgf14 by kinases**

Several studies show that Fgf14 controls the function of the Na-channels by strongly binding to the intracellular C-terminus of the α subunits of Na1.1, 1.2, and 1.6 [28,46] and that this complex localizes at the AIS [25]. In particular, Fgf14 regulates cellular targeting of Na channels at the AIS [26,34] through a glycogen synthase kinase 3 (GSK3) -dependent signaling pathway [34,47], which may modulate neuronal excitability [34,47]. GSK3 has recently been shown to play key roles in the regulation of axonal transport and cytoskeletal assembly during axonal growth, modulating neuronal polarization [49,50] and synaptic plasticity [51,52]. Fgf14 and the intracellular domains of Na-channels contain several predicted GSK3 phosphorylation motifs [34]. Pharmacological inhibition of GSK3 reduces the assembly of the Fgf14-Na-complex, modifies Fgf14-dependent regulation of Na+ currents, and alters the subcellular distribution and co-localization of Fgf14-Na-complex leading to reversal of its axonal-dendritic polarity in hippocampal neurons [34].

The expression of Fgf14 in heterologous cells leads to inhibition of peak amplitude of Na currents encoded by Na1.6 and Na1.2 channel isoforms [27] and these changes are likely to result from modulation in the number of cell surface available channels [25-26].
GSK3 inhibitors antagonize Fgf14 modulation of Na.1.2 rescuing Na+ current amplitudes back to control levels and antagonize the action of Fgf14 on Na.1.6 kinetics, with no detectable effects on Na.1.2 biophysical properties [34].

In hippocampal neurons treated with the GSK3 inhibitor CHIR99021, Hsu et al.,[47] found a significant depolarizing shift in action potential threshold, suggesting that inhibition of GSK3 affects neuronal excitability by shifting of the action potential threshold to a more positive value, likely through modulation of Na-channel function. Since Fgf14 is rich in potential motifs critical for cellular trafficking, and the Na-channel C-tail contains endocytic di-leucine motifs that are required for its axonal compartmentalization [53], Shavkunov et al.,[34] suggested that the uncoupling effect of GSK3 inhibitors on Fgf14- Na-channel complex could interfere with cellular trafficking of Na-channels, leading to loss in axonal polarity, and could prevent the accessibility of the channel C-tail di-leucine motifs, blocking channel endocytosis in the dendrites and limiting its compartmentalization in the axon [36].

In addition to GSK3 pathway, Hsu et al.,[47] also identified other kinase pathways modulating the Fgf14- Na.1.6 channel complex assembly, including the Wee1 kinase, the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway, protein kinase C, c-Jun N-terminal kinases (JNK), cyclin-dependent kinase 4 (Cdk4), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and eukaryotic elongation factor-2 kinase (eEF2K). Inhibitors targeting these kinases exert effects of opposite direction and different magnitude on the Fgf14-Na.1.6 complex assembly. For example, the Wee1 inhibitor I, a blocker of the serine/threonine kinase Wee1 implicated in the control of neuronal polarity [54], leads to an increase in Fgf14-Na.1.6 complex assembly. Similarly, inhibition of Akt, a negative regulator of GSK3, increases Fgf14-Na.1.6 complex assembly, and LY294002, a small molecule inhibitor of PI3K, which activates Akt, also increases Fgf14-Na.1.6 complex formation, supporting a PI3K/Akt/GSK3 directionally-mediated signaling cascade [47]. On the other hand, they found that blockers of the inhibitor of NFkB (IkB)-NFkB pathway implicated in neuronal plasticity, survival, and injury [55-56], strongly inhibit Fgf14-Na.1.6 association, as also found by Shavkunov et al.,[46], similarly to the effects of the inhibitors of eEF2K, a calcium-calmodulin dependent kinase involved in stabilization of dendritic spine morphology and plasticity [57-58]. A reduction of Fgf14-Na.1.6 complex assembly has also been induced by the p38/mitogen-activated protein kinase (MAPK) inhibitor PD169316 as reported by Shavkunov et al.,[46].

Recent data suggest a critical role of casein kinase 2 (CK2) in controlling neuronal excitability. In neurons, CK2, a serine/threonine protein kinase, acts as a priming kinase for GSK3, enhancing GSK3 phosphorylation and amplifying the GSK3 signaling pathway [51]. CK2 phosphorylates Na-channels at the specific residues that are within the ankyrin-G binding site [59-60], modulating trafficking to and stability of Na-channels at the AIS [61]. Prolonged inhibition of CK2 activity disrupts Na-channels localization at the AIS and impairs formation of neuronal polarity in immature cultured hippocampal neurons [62]. CK2 accumulation at the AIS depends on expression of Na.1 channels, and CK2-mediated phosphorylation contributes to Na.1 clustering at the AIS [59,60], taking part in the regulation of neuronal excitability.

Hsu et al.,[63] demonstrated a critical role of CK2 in phosphorylating Fgf14 and in controlling Fgf14 assembly with Na-channels. They showed that CK2
phosphorylates Fgf14 at S228 and S230 in vitro and its inhibition by TBB (4,5,6,7-tetrabromobenzotriazole) rapidly abolishes the Fgf14-Na.1.6 interaction and reduces the ability of Fgf14 to bind to Na.1.6 and Na.1.2 in a heterologous system. In neurons TBB reduces Fgf14 expression affecting its distribution in the soma, axons and dendrites in a development-dependent mode. The reduction of somatic Fgf14 expression is more evident at early developmental stages in vitro compared to mature neurons with established polarity. In addition, the effect of CK2 inhibition in disrupting Fgf14 polarity is more pronounced at an earlier time for mature axons as compared with mature dendrites; this delay may be due to temporary somatic sequestration and/or slower diffusion of Fgf14 from the somatic to the dendritic compartments. In hippocampal neurons, TBB reduces the amplitude of Na+ currents and induces a hyperpolarizing shift in voltage dependence of steady-state inactivation of NaChannels. In hippocampal slices, CK2 inhibition disrupts intrinsic excitability of pyramidal neurons by increasing spike current threshold and impairing neuronal excitability, a phenotype occluded by genetic deletion of Fgf14.

The effects of CK2 inhibition on Fgf14 expression and distribution and on sodium currents suggest a combined effect of the kinase in the regulation of targeting as well as the biophysical properties and number and/or availability of the NaChannels, as the result of a reduced Fgf14-Na+ channel interactions.

Role of Fgf14 in Intrinsic Membrane Excitability

Excitability defects in hippocampus

As previously described, the Fgf14 protein is highly expressed in hippocampal neurons, mainly at the AIS where it is coupled directly to the C-terminus of Na.α subunits [25-28], modulating neuronal excitability. Expression of Fgf14 with missense mutations reduces Na.α subunit expression at the AIS, attenuates Na+ channel currents, and decreases the excitability of hippocampal neurons [26]. In these neurons in vitro, the Fgf14 N-terminus is required for AIS targeting and functional regulation of NaChannels, with different effects mediated by the two N-terminal alternatively spliced Fgf14-1a and Fgf14-1b variants [27]. Several kinases regulate the interaction of Fgf14 with NaChannels and control hippocampal neuronal excitability, including GSK3 [34,47] and CK2 [63]. Inhibition of neuronal excitability has also been induced by the CK2 inhibitor TBB in CA1 hippocampal slices, with increased action potential threshold and reduced firing frequency [63].

Expression of Fgf14 in the AIS of CA1 γ-aminobutyric acid (GABA) -ergic parvalbumin (PV) interneurons has recently been reported by Alshammari et al., [38]. Interestingly, they found a significant reduction of GABAergic PV interneurons in Fgf14-KO mice, associated with decreased expression of presynaptic GABAergic markers glutamic acid decarboxylase 67 (GAD67) and vesicular GABA transporter (VGAT) in CA1 pyramidal neurons. In Fgf14-KO mice the remaining inhibitory PV interneurons showed a reduced firing of action potentials (longer inter-event intervals in distribution of spontaneous inhibitory postsynaptic currents frequency in CA1 pyramidal neurons) associated with pre- and postsynaptic modifications at GABA synapses (shift in frequency and amplitude distribution of miniature inhibitory postsynaptic currents, respectively). In addition, mice with genetic deletion of Fgf14 exhibited a reduction in both slow and fast gamma frequency oscillations in the CA1 region that route flow of information from the medial entorhinal cortex, an area that provides information about the animal’s current position, and from CA3, a hippocampal subfield essential for storage of such information [64-66]. Thus, a reduction in both slow and fast gamma implies that both intra-
and extra-hippocampal synaptic inputs might be compromised, possibly reflecting more widespread anomalies in Fgf14-KO brains, arising from the prefrontal cortex-thalamic-hippocampal loop [67]. Since PV interneurons represent the majority of GABAergic inputs in the CA1 and GAD67 and VGAT are essential for the synthesis and loading of GABA at presynaptic terminals, Alshammari et al., [38] suggested that Fgf14 genetic deletion might impair hippocampal inhibitory transmission, resulting in an unbalancing of the excitatory/inhibitory tone of cortical networks [68].

The functional integrity of GABA circuits is critical for cognitive processes in the brain and desynchronized network activity is associated with cognitive impairment in several psychiatric disorders, including schizophrenia and bipolar disorders [69,70]. Behavioral studies showed that Fgf14-KO animals exhibit cognitive deficits in reference memory-related capabilities [17] and impaired spatial working memory [38], without significant deficits in other non-spatial tests such as step-through passive avoidance and contextual fear [17]. These findings suggest that functions in brain areas such as hippocampal CA1 and CA3 that are important for spatial learning and memory are compromised in Fgf14-KO mice, while regions that are involved in contextual learning tasks result less affected.

Alshammari et al., [71] reported functional changes in the dentate gyrus (DG) circuit of Fgf14-KO animals. In particular they observed a reduced minimal threshold response and impaired paired-pulse facilitation at the perforant path to DG inputs, suggesting a disrupted synaptic connectivity associated to impaired neurogenesis. These phenotypes suggest that altered neurogenesis might lead to an over-excitatable DG circuit with neurons locked in an immature stage, more susceptible to develop an epileptic-like status [72-74]. Decreased threshold for seizure induced by cocaine and significantly shorter latency to seizure induced by pentylenetetrazole were reported in Fgf14-KO mice by Wang et al., [5], associated with ataxia and a paroxysmal hyperkinetic dyskinesia.

**Excitability alterations of cerebellar neurons**

Electrophysiological studies have provided important information on how the lack of Fgf14 can influence spontaneous firing and membrane excitability of cerebellar neurons. Goldfarb and colleagues [18] were the first investigators showing a reduced intrinsic excitability of cerebellar granule cells in Fgf14-KO mice. They demonstrated, by current clamp recordings, that these cells were unable to sustain high action potential firing frequencies with injection of moderate to strong depolarizing current [18]. Regarding PCs of Fgf14-KO mice, the majority (82%) of them lacked spontaneous firing, in contrast to wild-type PCs that were almost all (93%) firing under the same experimental conditions [32]. Similarly to Fgf14-KO granule cells, silent PCs were unable to sustain evoked repetitive firing [32]. Similar results were obtained by the acute Fgf14 knock-down in vivo [19]. Bosch et al., [19] performed stereotaxic intracerebellar injection of adeno-associated virus containing Fgf14-shRNA in the PC layer. The authors reported that more than 80% of the non-transfected cells were spontaneously active, while more than 80% of the transfected cells were silent [19]. Moreover, as in PCs of Fgf14-KO mice [32], PCs in which Fgf14 was knocked-down were unable to sustain repetitive firing evoked by prolonged current injection [19]. In accord with these studies, Yan et al., [75] showed that, in cultured PCs, shRNA knock-down of Fgf14 resulted in an increased current threshold to evoke an action potential and current injection evoked fewer action potentials [75].
It is important to note that, in current clamp experiments, in which the voltage membrane was manually held at hyperpolarizing values (-60 or -70 mV) the analysis of single action potential waveform, input resistance and voltage membrane revealed no changes [19,32,75].

These results, taken together, point to an important role of Fgf14 in regulating membrane excitability in cerebellar neurons. However, a normal spontaneous and evoked firing of PCs with either Fgf14-KO or Fgf14-knockdown was restored by a brief hyperpolarization of the membrane [19]. This suggests that Fgf14 affects the voltage-dependent properties of Na currents. Indeed, voltage clamp experiments revealed a significant hyperpolarizing shift from -58 to -71 mV in the \( V_{\text{rr}} \) of Na-steady-state inactivation in Fgf14-KO PCs [19]. A similar result was also reported by Yan et al., [75] in cultured PCs silenced with shRNA for Fgf14. A shift to the left of the steady-state inactivation curve causes a reduction in Na-channels availability at the resting potential of PCs. As a consequence, Fgf14 is necessary for Na-channels to recover from inactivation at the resting potential. In fact, membrane hyperpolarization resulted in a rescue of the repetitive firing of Fgf14-knockdown PCs [19].

The PC firing impairment following Fgf14 knockdown was associated with significant motor deficits [19]. Since in these experiments Fgf14 expression was selectively reduced only in the cerebellar cortex, in this case the motor deficits can be attributed to the suppression of Fgf14 function in this structure. In fact, most patients with SCA27 display ataxia as the dominant symptom. The results of Bosch et al., [19] indicate that ataxia is due to a deficiency of Fgf14 in the cerebellar cortex. It is very likely that the suppression of Fgf14 in the cerebellar cortex is the mechanism of ataxic symptoms in both SCA27 patients and Fgf14-KO mice. Furthermore, mice with a cerebellar cortex-selective knockdown of Fgf14 lack other types of neurologic symptoms that are present in global Fgf14-KO animals, like paroxysmal dystonia and cognitive deficits. Therefore, the latter symptoms should be attributed to the absence of Fgf14 in extracerebellar regions of the central nervous system, in agreement with the dependence of dystonia from basal ganglia, and of cognitive functions from the cerebral cortex. Consistently with Fgf14-KO mice, extracerebellar symptoms have been described also in SCA27 patients, with the only difference that in the latter case the genetic alteration of \( FGF14 \) was not homogeneous. For this reason also the symptoms were not homogeneous in SCA27 patients, with some cases in which ataxia and cognitive deficits were prevalent [7] and others in which the only symptom was a paroxysmal dyskinesia [9] or even a combination of ataxia, cognitive deficits and dyskinesia [8]. More intriguing is a case with episodic ataxia, triggered by fever [12]. This feature has not been modeled so far, but the temperature dependence is in line with the high sensitivity of Na channels to this parameter [13-14].

In conclusion, the selective presence of ataxia in the model with a selective knockdown of Fgf14 in the cerebellar cortex suggests that the integrity of \( FGF14 \) in the cerebellum is essential to prevent this symptom, while cognitive deficits and dystonia are due to alterations of \( FGF14 \) function in other brain regions, like basal ganglia and cerebral cortex.

**Fgf14 Control of Neurotransmitter Release**

In addition to the effects exerted by binding to Na channels, Fgf14 has been shown to also affect synaptic transmission [76]. In the study by Yan et al., [76], in mixed cerebellar rat primary cultures Fgf14 expression was knocked-down by shRNA. Under these conditions, Ca\(^{2+}\) currents recorded from granule cells were suppressed to about half of their normal amplitude. The main Ca\(^{2+}\) channel of granule cells,
Cav2.1, expressed in a heterologous system, showed larger currents when it was co-expressed with Fgf14. Yan et al., [76] showed that such larger current was due to an increased number of Cav1.2 channels. Since a main function of calcium channels in neurons is to trigger neurotransmitter release, excitatory postsynaptic currents (EPSCs) evoked by stimulation of a granule cell were recorded in PCs [76]. Synaptic transmission was reduced by more than 80% relative to control amplitude. This effect was due to a presynaptic mechanism, as shown by the decreased synaptic vesicle turnover and by short-term synaptic plasticity, both of which are Ca²⁺ dependent. In cultured granule cells, which endogenously express Fgf14, transfection with Fgf14b<sup>F160S</sup>, bearing a SCA27 mutation, suppressed both Ca²⁺ currents and synaptic transmission to PCs. Moreover, transfection of granule cells with wild-type Fgf14 further increased Ca²⁺ currents, suggesting that in cultured granule cells this effect of Fgf14 is not saturated [76]. These results indicate that Fgf14 is important to attain the normal amplitude of Ca²⁺ currents involved in the release of neurotransmitter from presynaptic fibers.

It is interesting to note that another function of intracellular forms of Fgf on calcium signaling was discovered in the heart. In fact, it has been shown that Fgf13 binds to the cardiac protein Junctophilin-2, involved in the coupling between T-tubules Ca²⁺ channels and sarcoplasmic reticulum RyR<sub>2</sub> channels [77]. Knockdown of Fgf13 reduced the number of Ca²⁺ channels in the sarcolemma and thereby the amplitude of Ca²⁺ currents and in addition also suppressed Ca²⁺-induced Ca²⁺ release mediated by RyR<sub>2</sub> channels [77].

The severe synaptic deficit described by Yan et al., [76] at the granule cell-PC synapse, if present also in vivo, might contribute to motor deficits. In order to test this hypothesis, the granule cell-PC synapse was studied in slices of cerebellar cortex from adult Fgf14-KO mice [78]. EPSCs evoked by stimulation of parallel fibers were reduced to about half size relative to wild-type controls. The analysis of short-term synaptic plasticity and of asynchronous miniature EPSCs showed that the mechanism was presynaptic. In order to confirm the presence of a presynaptic alteration, the specific marker of parallel fiber synaptic varicosities vesicular glutamate transporter 1 (VGLUT<sub>1</sub>) was measured, revealing a reduction in Fgf14-KO mice. The main postsynaptic glutamate receptors of PCs, GluA<sub>2</sub> and mGlu<sub>1</sub>, were intact, confirming that the impairment of synaptic transmission was entirely dependent on neurotransmitter release from granule cell axons [78]. The mechanism responsible for the potentiating action of Fgf14 on neurotransmitter release was not further investigated, so that it is possible that the normal amplitude of granule cell Ca²⁺ currents is dependent on Fgf14. However, a recent proteomic study on cerebellar tissue did not reveal any binding partner of Fgf14 belonging to the Ca²⁺ channel complex [79]. However, it was found that Fgf14 binds to synaptotagmin 1 and 2 and to SNAP-25 [79], which are some of the main components of the Ca²⁺-triggered neurotransmitter release machinery. Therefore, an alternative hypothesis is that Fgf14 acts downstream of Ca²⁺ channels directly on the proteins controlling synaptic vesicle exocytosis. Future experiments are needed to test these two hypotheses.

**Functional Alterations in Other Brain Regions**

**Localisation of Fgf14 in the developing and adult brain**

Fgf14 gene expression is detectable in the mouse brain from embryonic day 12.5 (E12.5) and it is localized in the supraoptic area, septal area, thalamus and in the ventral lining of the Acqueduct of Sylvius and forth ventricle [6]. In order to assess the role of Fgf14 in the development of the nervous system, anatomical...
examination of different brain structures was performed by using different staining methods starting from embryonic life up to 3 months of age in wild type and in Fgf14-KO mice. Luxol Fast blue, silver staining and BrdU revealed that the organization of cell bodies, axon myelination, axonal growth and neuronal migration were not altered in Fgf14-KO mice demonstrating that, from the anatomical point of view, the brain structure is not affected by the lack of Fgf14 during development [5] and suggesting that this protein is not essential for neuronal development. An explanation for this may be related to the expression patterns of different Fgfs which are overlapping in the developing nervous system and which may give rise to functional redundancy and compensatory mechanisms [4,5].

The Fgf14 expression pattern in the mature brain is conserved across species, since RNA blot analysis from human brains and in situ hybridization with cDNA probes in mice showed the same distribution of the protein [5]. The highest levels of Fgf14 expression have been found in both human and mouse in the cerebellum and basal ganglia while significant levels were also expressed in the hippocampus, amygdala, cerebral cortex and thalamus [5,17].

**Basal ganglia involvement in SCA27**

Together with the cerebellum, the basal ganglia are the structures where the highest expression levels of Fgf14 have been described [5]. From about 1 month of age, Fgf14-KO mice show episodes of hyperkinetic movements that have been described as paroxysmal dyskinesia [5]. Since the most frequent cause of dyskinesia are basal ganglia disorders, it has been hypothesized that this motor disturbance might be due to a dysfunction of basal nuclei [5].

The basal ganglia, which comprise multiple subcortical nuclei, are associated with a variety of functions including: control of voluntary movements [80], procedural learning [81], routine behaviors or 'habits' [82], eye movements [83], cognition [84] and emotion [85]. The neuronal circuitry underlying the functional architecture of the basal ganglia involves inhibitory GABAergic pathways to the thalamus via the substantia nigra pars reticulata (SNr) and the globus pallidus (GP) and a positive glutamatergic feedback from the thalamus to the cortex. The SNr and GP complex, which gives inhibition to the thalamus, is indirectly modulated by a dopaminergic pathway coming from the substantia nigra pars compacta. This pathway modulates the excitatory inputs to the primary motor cortex, thus the final outcome is the regulation of movement [86]. It has been suggested that both hyperkinetic and hypokinetic movement disorders are related to impairment of basal ganglia function [87,88] involving alterations of the dopaminergic system [89,90].

In order to assess the role of dopaminergic neurons of the basal ganglia in SCA27-related hyperkinesia, anatomical investigation with specific markers has been performed in Fgf14-KO mice. Immunostaining for tyrosine hydroxylase (TH, the enzyme catalyzing the production of the dopamine precursor L-DOPA), for dopamine and its metabolites revealed that dopaminergic neurons in the substantia nigra pars compacta and in the ventral tegmental area (VTA), the two major structures involved in motor diseases such as Parkinson’s disease, appear normal [5]. Furthermore no differences between wild type and mutant mice were found in the striatum, in the SNr and in the VTA area providing evidence that dopaminergic neurons and their projections are structurally normal. Moreover, the levels of dopamine and its metabolites were also indistinguishable in Fgf14-KO compared to wild-type mice [5]. These findings suggest that the role of Fgf14 in basal ganglia function is not due to alterations of the dopaminergic
In a different mouse model showing paroxysmal dystonia, functional rather than structural, alterations of the basal ganglia dopaminergic system have been demonstrated [91,92]. In order to assess a similar feature in Fgf14-KO mice, locomotor activity tests were performed in mice treated with cocaine (an inhibitor of dopamine re-uptake), amphetamine (which facilitates dopamine release) and specific dopamine agonists. Cocaine and amphetamines increase the level of synaptic dopamine inducing hyperactivity in mice, rats and humans [93-95]. Different doses of cocaine ranging from 2 to 10 mg/kg were delivered to Fgf14-KO and wild-type mice and locomotor activity was observed. At all tested doses no hyperactivity was detected in Fgf14-KO mice while in wild-types hyperactivity was observed already at 2 mg/kg. Following amphetamine administration (2 or 4 mg/kg) in Fgf14-KO mice hyperactivity was significantly reduced at both doses [5]. A similar lack of locomotor hyperactivity induced by cocaine was observed in mutant mice lacking D1 class receptors [96]. In these mice, amphetamines caused locomotor hyperactivity starting at doses of 5mg/kg, suggesting that amphetamines are more effective than cocaine in inducing hyperactivity and that this effect is mediated by D1 receptors. Thus, it can be hypothesized that the reduced locomotor activity in Fgf14-KO mice might be due to a diminished response of D1 receptors in striato-nigral neurons [5]. However, the administration of a D1 receptor agonist caused a marked locomotor hyperactivity in Fgf14-KO mice, not significantly different from the response of wild-type controls [5]. Surprisingly, the response to the administration of a dopamine D1 receptor agonist, which consists in a strong decrease of locomotor activity in wild-type mice, was absent in Fgf14-KO animals [5]. The role of dopamine and of basal ganglia in this model of SCA27 was not investigated further, so that the contribution of basal ganglia in motor symptoms of Fgf14-KO mice remains uncertain.

**Functional correlates of SCA27 in hippocampus and amygdala**

The amygdala and hippocampus have been demonstrated to be involved in a variety of behavioral tasks and often these two structures are synchronized via theta oscillations in humans and mice [97-99]. Individuals affected by SCA27, besides sensorimotor symptoms, exhibit impaired cognitive capabilities, which include tasks where the hippocampus and the amygdala are demonstrated to play a pivotal role [100-102]. Fgf14 is significantly expressed in these two structures both in humans and mice [5,17]. More in detail, in situ hybridization in the mouse brain has shown that Fgf14 transcripts are present in principal cells and interneurons in the CA1, CA3 regions and DG of the hippocampus [33], in the dorsolateral and anterior basolateral amygdaloid nuclei and in the basolateral amygdala [17]. Furthermore long term but not short term potentiation of the Schaffer collateral-CA1 synapse in hippocampal slices from adult mice was impaired in Fgf14-KO mice [33] suggesting that Fgf14 may play a functional role in long term synaptic changes which have been proposed to underlie memory and learning processes [103-105].

Based on these observations, in order to assess the functional role of Fgf14 in cognitive tasks involving the hippocampus and amygdala, focused behavioral experiments were performed in Fgf14-KO mice where spatial learning, passive avoidance and conditioned fear were tested [17]. Long-term spatial memory was tested by the Morris water navigation task, mostly used with rodents, where a submerged platform is located in a pool filled with an opaque liquid. After a few trials, where the animal swims randomly in the pool until it can step on a platform, the animal learns where...
the platform is and swims towards it with shorter escape latencies [106]. The performance to this task has been linked to hippocampal long term potentiation and NMDA receptor function, making it a key technique in the investigation of hippocampal circuitry [107,108]. In the Morris water maze task, Fgf14-KO mice showed impairment in the acquisition phase. However, in the probe trial used to test long-term retention, their performance was not different from that of their wild-type littermates [17]. Only in a reversal learning test performed two months later Fgf14-KO mice showed a deficit in the probe trial [17]. Taken together, these results suggest that Fgf14-KO mice have a mild impairment of long-term spatial memory.

To further investigate a possible role of Fgf14 in different forms of learning, Fgf14-KO mice were also tested on a step-through passive avoidance task. In this task a light chamber and a dark chamber are connected with a sliding door. The rodent is placed in the illuminated chamber and when the door opens it moves into the dark one, which represents a safer place. However, a foot shock is given when the animal steps into the dark chamber. After a few trials the animal learns to avoid stepping into the dark chamber and the latency between the presence in the light chamber and in the dark one (step through latency) becomes longer and longer until the animal doesn’t enter the dark chamber anymore. In order to test the acquisition and the extinction of this behavior, animals were tested 24h after the trials and the time spent before entering the dark chamber (where the foot shock was not delivered anymore) was measured. Fgf14-KO mice showed no significant difference compared to wild types [17]. Similar results were observed when the same groups of animals were submitted, 2 months later, to fear conditioning training where both responses to auditory and contextual cues were not significantly different between Fgf14-KO and wild-type mice [17].

In rodents, short-term working memory depends from hippocampus and prefrontal cortex [109]. Spatial working memory was significantly impaired in Fgf14-KO mice when tested in an eight arm radial maze [38]. Taken together, these results suggest that Fgf14-KO mice have mild deficits of some cognitive functions including working memory and long-term spatial memory, while other cognitive aspects are intact.

Possible Role of Fgf14 in Psychiatric Disorders

Understanding the molecular mechanisms underlying psychiatric disorders is a key step for the development of effective pharmacological treatments. However the cellular pathways involved in this kind of diseases depend on a variety of factors, which cannot be identified solely in molecules and receptors but rather depend on a complex combination of environmental and genetic factors, which may favor the establishment of disrupted cellular and molecular functions underlying mental disorder. In this context, recent findings have put in evidence the importance of neuronal excitability as a substrate for the correct circuitry establishment and function both during development and in the adult [110-113]. The AIS is a key neuronal compartment in determining neuronal excitability because it is responsible for action potential initiation. In neurons, the local density of Na⁺ channels, the biophysical properties of K⁺ channels, as well as the length and diameter of the AIS determine action potential firing. Recently it has been demonstrated that a dynamic regulatory process occurs at the AIS on a time scale of hours or days in order to tune neuronal excitability and adapt to the surrounding neuronal network to comply with particular physiological functions [113]. In contrast, the activation of metabotropic receptors

modulates the properties of ion channels expressed at the AIS within seconds and consequently produces fast adjustments of neuronal excitability [114]. This plasticity is thought to play important roles in physiological functions such as cognitive and behavioral tasks [115,116] and disruption of this mechanism has been associated with psychiatric disorders such as schizophrenia and bipolar disorder [117,118].

As previously mentioned, Fgf14 is located at the AIS of many types of neurons, and its loss results in diminished excitability of different neuronal populations in the cerebellum and hippocampus. Altered neuronal excitability has been correlated with psychiatric disorders [119,120]. Immunoreactivity for Fgf14 has been described in developing granule cells in the mature hippocampal DG where it plays a fundamental role in adult neurogenesis guaranteeing the transition from late immature to early mature neurons [71]. Disruption of Fgf14 function in Fgf14-KO mice impairs synaptic integration of neurons in the hippocampal circuitry [38]. Alteration of the maturation process in the DG has been proposed as underlying disturbances in mental health, as markers of immature DG have been observed in post-mortem analysis of brains from patients with schizophrenia/bipolar disorder [121].

Conclusions

The results reported in this review clarify possible roles of FGF14 in complex brain disorders characterized by cerebrocerebral neuronal networks unbalance. Figure 1 shows a schematic, detailing the roles of FGF14 in the modulation of brain functions. Regarding the relationship between FGF14 and SCA27, data from genetic analysis of patients suggest a loss-of-function mechanism. The results obtained by a second type of approach, namely in vitro experiments, show that the main molecular role of FGF14 is to modulate the function of Na+ channels, with isoform-specific effects. In addition, FGF14 is required to maintain a proper neurotransmitter release at some synapses. A third line of evidence derives from studies of the Fgf14-KO animal model. In Fgf14-KO mice, the ataxic motor dysfunction can be attributed to at least three mechanisms, which combine their effects: i. impairment of action potential generation in cerebellar granule cells; ii. deficiency of action potential generation in cerebellar Purkinje cells; iii. deficit of neurotransmitter release from parallel fibers, which are the axons of cerebellar granule cells. Putting together the knowledge derived from genetic and in vitro studies with the data acquired from the knockout animal model, it can be hypothesized that the ataxic symptoms of patients with SCA27 can be attributed to a deficiency of FGF14 function, leading to impairment of neuronal signaling in the cerebellar cortex.

A new view emerging in the last few years suggests that FGF14 might be involved in psychiatric conditions including schizophrenia, depression, bipolar disorder, anxiety, drug addiction, eating disorders. In fact, FGF14 is expressed in specific types of neurons in the key neural structures involved in these psychiatric
disorders. We propose that, in addition to neurologic symptoms like cerebellar ataxia and paroxysmal dyskinesia, FGF14 variants or mutations might participate to the development of a variety of psychiatric conditions.

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