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Spontaneous seizures in *Kcna 1*-null mice lacking voltage-gated Kvl.1 channels activate Fos expression in select limbic circuits

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Abstract

Mice lacking voltage-gated Kv1.1 channels as a result of deletion of the *Kcnal* gene are an extensively utilized genetic model of human epilepsy and sudden unexpected death in epilepsy because of their frequent seizures and genotypic-phenotypic similarity to the human condition. Ictal behaviors, electrophysiological recordings, and gene expression studies suggest limbic circuits are critical for epilepsy in *Kcna1*-null mice, but the exact brain networks recruited by seizures remain unknown. In this study, Fos protein expression patterns were used to map limbic brain regions with increased neuronal activity at baseline and during spontaneous seizures in *Kcna1*-null mice by comparing seizing and non-seizing knockouts and wild-type controls. Basal Fos levels were unchanged in non-seizing knockout mice compared to wild types for all brain regions examined except the dentate gyrus granule cell layer which exhibited a significant decrease in Fos-positive cells. Following seizures, *Kcna1*-null brains exhibited significantly increased Fos labeling in the basolateral amygdala and the dentate hilus region, but not in other principal cell layers of the hippocampal formation. The selective Fos activation in the amygdala following seizures suggests that extra hippocampal limbic circuits may be critically involved with seizure generation or spread in *Kcna1*-null mice.

Keywords

amygdala; epilepsy; Fos; hilus; Kv1.1 channel; seizure

Kv1.1 voltage-gated potassium channel a-subunits, encoded by the *Kcnal* gene, act to dampen neuronal excitability by regulating action potential propagation and shape, repetitive firing properties of neurons, and neurotransmitter release (Glasscock *et al.* 2012; Jan and Jan 2012). Mutations in *KCNA1* lead to neurological disorders associated with neuronal hyperexcitability. In patients, mutations in *KCNA1* have been linked to episodic ataxia type 1, neuromyotonia, epilepsy, and sudden unexpected death in epilepsy (Browne *et al.* 1994; Zuberi *et al.* 1999; Kinali *et al.* 2004; Klassen *et al.* 2014). Mice lacking Kv1.1 channels as a result of targeted deletion of the *Kcna1* gene exhibit phenotypes similar to patients,

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conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

including severe epilepsy characterized by frequent spontaneous convulsive seizures that occur several times daily and often lead to sudden death (Smart *et al.* 1998; Glasscock *et al.* 2010). The high frequency of seizure activity in *Kcna1* -null mice coupled with their genotypic-phenotypic similarity to human temporal lobe epilepsy has made them a preferred model for the study of anti-seizure drug and cellular gene therapies, as well as genetic modifier interactions in epilepsy (Glasscock *et al.* 2007; Baraban *et al.* 2009; Holth *et al.* 2013; Bomben *et al.* 2014).

Although the *Kcna1* -null model is widely used to study epilepsy, little is known about the neuronal circuitry responsible for the generation and spread of seizures in these mice. Seizures in *Kcna1* -null mice have been hypothesized to involve limbic brain circuits based on ictal behaviors, electrophysiology, and channel expression. The seizure-associated behaviors in *Kcna1* -null mice resemble the well-characterized kainate and kindling-induced models of temporal lobe epilepsy in rodents, which involve amygdala and hippocampal neurocircuits (Smart *et al.* 1998; Rho *et al.* 1999; Wenzel *et al.* 2007). In *Kcna1* -null mice, seizure-like EEG patterns sometimes occur in hippocampal depth electrodes immediately preceding ictal activity at the cortical surface, suggesting that some seizures may originate in limbic structures (Wenzel *et al.* 2007). In brain slice recordings from *Kcnal* -null mice, the CA3 hippocampal region exhibits abnormal burst firing under conditions of high extracellular K^+ concentrations, $GABA_A$ antagonism, and electrical stimulation suggesting the hippocampus is particularly susceptible to epileptiform discharges in this model (Smart *et al.* 1998; Lopantsev *et al.* 2003; Glasscock *et al.* 2007). The increased *in vivo* and *in vitro* hippocampal excitability in *Kcnal* -null mice correlates with the distribution pattern of Kv1.1 subunits, which are normally expressed at high levels in the axon tracts of the hippocampus and dentate gyrus, including the perforant, mossy fiber, and Schaeffer collateral pathways, and in the cell bodies of some hilar interneurons (Wang *et al.* 1994; Wenzel *et al.* 2007). Taken together, limbic structures appear to be critically involved in the generation or spread of spontaneous seizures in *Kcnal* -null mice; however, the identity of the exact brain circuits activated by seizure activity in these mice remains unknown.

A widely used biochemical technique to map neuronal activity during seizures is immunohistochemical visualization of Fos protein expression. Fos protein, encoded by the immediate early gene *c-fos,* is rapidly and transiently translated in response to intense synaptic excitation, such as occurs during seizures, making it a useful biomarker of neuronal activation (Morgan *et al.* 1987). In neurons, a stimulus induces production of Fos protein, which combines with Jun, another immediate early gene protein, to form a heterodimeric transcription factor that regulates expression of late-response genes (Herrera and Robertson 1996). Fos levels peak transiently about 2 h after a neuron-activating stimulus such as intense seizure activity (Morgan *et al.* 1987). Fos labeling has been used extensively to identify neurons that are activated during chemically-, electrically-, or stress-induced seizures in rodent models (Herrera and Robertson 1996). However, very few studies have utilized Fos imaging to measure neuronal activation following spontaneous seizures in genetic models of epilepsy, probably owing to the inherent difficulty associated with collecting tissues within the short-lived temporal window of Fos expression when seizure occurrence is unpredictable.

Here, Fos labeling was utilized to identify brain regions activated by spontaneous seizures in the *Kcna1* -null mouse for the first time. *Kcna1* -null mice were video monitored in their home cages to identify behavioral seizures. Fos immunohistochemistry was then performed to map neuronal activity in brain sections, which was correlated with the presence or absence of behavioral seizures in the 4 h immediately preceding tissue harvesting. Specifically, we tested whether knockouts exhibit basal increases in Fos labeling in particular neuronal subsets that might reflect a propensity for seizure activity. We also examined whether Fos expression is increased in discrete brain regions following spontaneous behavioral seizures, suggesting neuronal activation during seizure generation or spread.

Materials and methods

Animals and genotyping

Kcna1 $^{-/-}$ knockout (KO) mice carry null alleles of the *Kcna1* gene (chromosome 6) resulting from targeted deletion of the open reading frame, as previously described (Smart *et al.* 1998). The mice were bred and maintained on a Tac:N:NIHS-BC genetic background. Animals were housed at 22°C, fed *ad libitum,* and submitted to a 12 h light/dark cycle. All procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH), as approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center-Shreveport.

For genotyping, genomic DNA was isolated by enzymatic digestion of tail clips using Direct-PCR Lysis Reagent (Viagen Biotech, Los Angeles, CA, USA). The genotypes of *Kcna1* mice were determined by performing PCR amplification of genomic DNA, using allele-specific primers: a mutant specific primer (5'-CCTTCTAT CGCCTTCTTGACG-3'), a wild-type specific primer (5'-GCCTCTG ACAGTGACCTCAGC-3'), and a common primer (5'-GCTTCAGG TTCGCCACTCCCC-3'). The PCR yielded amplicons of ~337 bp for the wild-type allele and ~475 bp for the null allele.

Video monitoring of spontaneous seizures

Individually housed KO mice were video monitored in their home cages using infrared-equipped digital network cameras (DCS-942L, D-Link, Fountain Valley, CA, USA). Digital video was recorded continuously for the 24-h period immediately preceding euthanasia and tissue collection. Videos were reviewed offline to identify spontaneous convulsive seizures, which exhibit easily identifiable behavioral features similar to kindling and kainate rodent models of temporal lobe epilepsy, as described in the Results. Seizures were scored for severity using the Racine scale (1, for mouth and facial movement; 2, head nodding; 3, forelimb clonus; 4, rearing with forelimb clonus; and 5, rearing and falling with forelimb clonus) (Racine 1972). Seizure durations were approximated by measuring the time from the onset of the seizure (characterized by initial immobility coupled with head nodding) to the cessation of behavioral convulsion.

Immunohistochemistry

Age-matched (1–3 months old) KO and *Kcna1++* (WT) mice of both sexes were killed within 30 min of the end of video monitoring by inhaled isoflurane overdose and intracardially perfused with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. Animals were always killed at a similar time of day during the light phase (between 10:00 a.m. and 2:00 p.m.) to prevent potential confounding effects because of diurnal variation in neuronal activity. Following perfusion, the brains were removed and post-fixed for 2 h followed by overnight cryoprotection in 30% sucrose in PBS. Brains were then embedded in optimum cutting temperature medium and frozen at —20°C until sectioned. Coronal sections (50 im thick) were cut using a Leica cryostat maintained at —20°C and collected in PBS for free-floating immunohistochemistry. Sections were blocked for 1 h in antibody vehicle (3% bovine serum albumin, 0.3% Triton X-100 in PBS) and then incubated overnight at 22°C with rabbit polyclonal anti-c-Fos antibody (Ab-5, 1 : 20 000; Calbiochem, La Jolla, CA, USA). After washing with PBS, sections were incubated with biotinylated anti-rabbit secondary antibody (1 : 750; Vector Laboratories, Burlingame, CA, USA) for 1 h. After washing again with PBS, sections were incubated in avidin-biotin peroxidase complex (ABC kit; Vector Laboratories) for 1 h. The sections were then developed using a 3,3'-Diaminobenzidine peroxidase substrate kit (Vector Laboratories); 3,3'-Diaminobenzidine reactions were always carefully timed to ensure all sections were equally treated. Sections were mounted serially on glass slides and air-dried overnight. The slides were dehydrated using an ethanol series (75%, 85%, 95%, 100%, 100%), cleared with Histo-clear II (National Diagnostics, Atlanta, GA, USA), and then cover-slipped.

Images were captured using an Olympus BX43 light microscope outfitted with an Olympus DP72 digital camera and cellSens software. Images were analyzed for Fos-positive labeling using ImageJ software (National Institutes of Health; Bethesda, MD, USA). Immunolabeled cells were counted using the Particle Analyzer tool in ImageJ and applying the same image, threshold, size, and circularity settings for each brain region. For each animal, cell counts were obtained from two to three non-consecutive sections between Bregma −1.2 to −1.4 mm for the basolateral amygdala (BLA) and piriform cortex (layer two) and between Bregma −1.3 to −1.9 mm for the dentate granule cell layer, dentate hilus region, and the CA1/CA3 pyramidal cell layers (Paxinos and Franklin 2013). Each brain region of interest was outlined and measured using ImageJ to calculate the area. The number of Fos-positive cells for each brain region was then expressed as cells per square millimeter. All immunohistochemistry tissue processing and cell counts were performed while the observer was blinded to genotype and seizure phenotype.

Statistical analysis

Data are expressed as means ± SEM. Prism 6 software for Windows (GraphPad, La Jolla, CA, USA) was used for analyses. One-way anova (a = 0.05) was used to evaluate cell counts, followed by Tukey's multiple comparisons test. Reported p values were generated from the multiplicity adjusted p values for each comparison as calculated by the Prism software.

Results

Behavioral seizure detection in knockout mice

To examine increases in Fos expression as a result of basal differences and seizure activity in *Kcnal*-null (KO) mice, three experimental groups were generated for comparisons based on genotype and seizure history: (i) seizing KO mice; (ii) non-seizing KO mice; and (iii) wild-type (WT) *Kcna1+ +* controls. Seizing and non-seizing KO mice were identified and scored by video monitoring knockout mice in their home cages to identify behavioral seizures during the 4-h period immediately preceding tissue harvesting. Seizures in KO mice exhibit easily identifiable behaviors facilitating accurate detection. As previously reported, the typical seizure in *Kcnal*-null mice usually lasts between 20 s to 2 min and includes the following pattern of behavioral progression, which is also characteristic of kainate and kindling models: initial immobility; tail extension; facial twitching, and head nodding; followed by unilateral and bilateral forelimb clonus; rearing and falling; and then synchronous forelimb and hindlimb clonus (Smart *et al.* 1998; Rho *et al.* 1999; Wenzel *et al.* 2007). These ictal behaviors in KO mice always correlate with seizure activity in electroencephalography (EEG) recordings (Smart *et al.* 1998).

Of the 13 KO mice examined in this study, 6 knockouts exhibited obvious behavioral seizures during the 4-h period immediately before tissue harvesting and were designated as the ‘seizing’ group. The observed seizures exhibited the same repertoire of behaviors as described above. Most seizures were intermediate in severity, not progressing past bilateral forelimb clonus (stage 3); however, 5 mice exhibited at least one higher grade seizure characterized by forelimb myoclonus with rearing (stage 4) or with rearing and falling (stage 5). Table 1 provides a summary of the seizures observed by animal in the seizing group, including behavioral severity, duration, and approximate onset time relative to tissue harvesting. The remaining 7 KO animals did not show obvious seizure behaviors during the 4 h prior to tissue harvesting and were designated as the ‘non-seizing’ group. Previous studies have shown that knockout mice occasionally exhibit electrographic seizures without obvious behavioral changes, raising the possibility that some seizures could have eluded detection (Smart *et al.* 1998). However, as described below, the Fos labeling in non-seizing *Kcnal* -null mice showed substantial overall similarity to wild-type controls but not to seizing *Kcnal* -null mice. This pattern suggests that either electrographic-only seizures were absent in the nonseizing group during the observation period or they were not sufficient to significantly activate Fos expression. Importantly, all animals were kept undisturbed in a quiet room in their home cages during video monitoring prior to euthanasia to prevent confounding effects of external stimuli, such as loud noises, novel odorants, or handling.

Basal Fos expression appears mostly unchanged in KO mice

Since KO mice exhibit frequent interictal EEG discharges (up to 100s per hour) and intrinsic neuronal hyperexcitability in brain slice recordings, we hypothesized that Kv1.1-deficient brains would show increased basal levels of cellular Fos expression in the absence of seizures. Quantification of Fos-labeled cells was performed in limbic structures, including the BLA (Fig. 1), piriform cortex (Fig. 2), dentate gyrus (granule cell layer and hilus; Fig. 3), and hippocampus (CA1 and CA3 pyramidal cell layers; Fig. 4). In WT control mice,

scattered Fos-positive cells were consistently observed in limbic and cortical regions. In non-seizing KO mice, the abundance of Fos-labeled cells was not significantly different from the levels observed in WT controls for all limbic regions examined, except for the dentate granule cell layer which exhibited a significant ~75% decrease relative to WT mice ($p = 0.005$; Fig. 3e). The lack of significant increases in Fos-labeling between non-seizing *Kcna1*$^{-/-}$ mice and WT controls in limbic regions suggests that intrinsic hyperexcitability and interictal activity as a result of Kv1.1-deficiency are not sufficient to substantially activate basal Fos protein expression.

Seizures activate Fos expression in select limbic regions in KO mice

Since Fos expression acts as a sensitive indicator of neuronal activity during chemically-or electrically-induced seizures, we hypothesized that Fos labeling could be used to detect brain regions recruited by spontaneous seizures in KO mice. To identify limbic structures that are abnormally activated by seizure activity, Fos labeling in seizing KO mice was compared to WT controls and to non-seizing KO mice. Overall, the brains of seizing KO mice exhibited increased numbers of Fos-labeled cells indicative of neuronal activation in several limbic regions compared to WT and nonseizing controls. In seizing KO mice, the BLA was the only limbic structure examined that exhibited a significant increase in Fos-labeling (three to fourfold) compared to both WT ($p = 0.005$) and non-seizing KO mice ($p = 0.002$; Fig. 1d). The hilus of seizing animals also showed drastically increased Fos-labeling of at least fourfold compared to WT ($p = 0.027$) and about threefold compared to non-seizing KO animals, but the latter comparison did not reach statistical significance ($p = 0.076$; Fig. 3d). The Fos-positive cells in the hilus are presumed to be predominantly inhibitory interneurons, but excitatory mossy cells also localize to this region (Freund and Buzsaki 1996). The piriform cortex of seizing KO mice exhibited a twofold increase in Fos-positive cells compared to WT and non-seizing KO mice, but these changes were not statistically significant ($p = 0.110$ and $p = 0.090$, respectively; Fig. 2d). The number of Fos-labeled cells in the dentate granule cell layer and the CA1 and CA3 pyramidal cell layers of seizing KO mice did not show significant differences compared to WT and non-seizing KO mice (Figs 3 and 4). Notably, 5/6 seizing KO mice and 1/7 non-seizing KO mice exhibited numerous intensely labeled Fos-positive cells in the stratum radiatum subfield of CA1 and CA3, which is home to several types of GABAergic interneurons (Fig. 4c and g) (Freund and Buzsaki 1996). In WT mice, sporadic Fos-labeled cells were rarely observed in the CA1/CA3 stratum radiatum layer (Fig. 4a and e). The robust Fos activation in the amygdala and hilus of seizing KO mice suggests these structures are critical components of the seizure circuitry in these animals. Furthermore, the increased Fos labeling in seizing KO mice compared to nonseizing KO mice suggests that seizures are responsible for the Fos activation and not baseline increases in neuronal activity.

Discussion

Previous work in *Kcna1* -null mice has focused on the hippocampus as the primary onset zone of seizures, but the seizure-related Fos pattern in KO mice suggests the possibility of an extrahippocampal contribution by the BLA. Although the role of the amygdala in epilepsy is not as well-recognized as the hippocampal formation, it has been implicated in

seizure initiation and spread in both patients and animal models (Kullmann 2011). Normally, the amygdala mediates emotional processing of sensory information and fear-related responses, learning, and memory (Sah *et al.* 2003). Anatomically, the amygdala possesses reciprocal connections with other limbic nuclei (including the hippocampus and piriform cortex), which can facilitate coactivation and seizure spread, respectively (Loscher and Ebert 1996; Davis and Whalen 2001; Sah *et al.* 2003). In patients, surgical resection of the amygdala has been used as an effective treatment for temporal lobe epilepsy (Feindel and Rasmussen 1991; Schramm 2008). Resected amygdala tissue from temporal lobe epilepsy patients shows the capacity for spontaneous interictal discharges when recorded *in vitro,* as well as alterations in neurotransmitter receptor densities, which together suggest evidence of disinhibition of amygdala circuitry that could contribute to seizure initiation (Graebenitz *et al.* 2011).

In rodent models, the amygdala exhibits extreme susceptibility to electrical kindling-induced seizures (Goddard *et al.* 1969). Similar to temporal lobe epilepsy patients, the amygdala in epileptic pilocarpine-treated rats exhibits spontaneous neuronal activity and altered inhibitory synaptic properties suggesting hyperexcitability (Benini and Avoli 2006). In addition, chronic spontaneous seizures in pilocarpine-treated rats induce Fos expression in a pattern similar to seizing KO mice, including Fos-labeling in the amygdala, piriform cortex, and interneurons of the hippocampus and dentate gyrus (Harvey and Sloviter 2005). However, the principal cells of the hippocampus and dentate gyrus remain largely Fos-negative, as in *Kcna1*-null mice, suggesting that spontaneous seizures in pilocarpine-treated rats originate outside of the hippocampus (Harvey and Sloviter 2005). At baseline, the significantly reduced Fos protein expression in the granule cell layer of non-seizing KO mice is consistent with reports of persistent down-regulation of basal *c-fos* mRNA levels in the dentate gyrus of young KO mice between the ages of P10-P34 (Rho *et al.* 1999). Similarly, chronically epileptic pilocarpine-treated mice also exhibit decreased Fos-labeling in granule cells between 4–8 h after a single spontaneous seizure, which is comparable to the postseizure interval of > 4 h in our non-seizing KO group (Peng and Houser 2005). Reductions in basal Fos levels may be because of a temporary refractory period for expression reinduction or a long-term adaptive response in a particular brain region (Morgan *et al.* 1987; Mello *et al.* 1996; Rho *et al.* 1999).

In addition to the Fos labeling patterns described in this work, previous neuropathological studies provide additional evidence for excessive activation of the amygdala and hilus during seizures in *Kcna1* -null mice. In KO mice experiencing status epilepticus, histological experiments reveal extensive neuronal cell death, terminal degeneration, and astrogliosis in the hilus, amygdala, piriform cortex, and hippocampus (Wenzel *et al.* 2007). Interestingly, however, neuronal architecture in the BLA nucleus is relatively preserved (Wenzel *et al.* 2007). Magnetic resonance imaging of *Kcna1* -null brains reveals significantly enlarged hippocampus and ventral cortex, including the amygdala and piriform region (Persson *et al.* 2007). *Megencephaly (mceph)* mice, which carry a mutation in *Kcna1* causing truncated and non-functional Kv1.1 channels, also exhibit spontaneous seizures coupled with brain enlargement in these areas (Diez *et al.* 2003; Persson *et al.* 2005). Brain overgrowth because of Kv1.1-deficiency correlates with seizure activity since seizure suppression by either

anticonvulsant therapy or genetic modifiers normalizes brain volume (Lavebratt *et al.* 2006; Holth *et al.* 2013).

Although Fos activation is a widely used biomarker of neuronal activity, the technique has some inherent limitations. Since Fos immunoreactivity provides only a snapshot of neuronal activity, it does not allow determination of the sequence of neuronal recruitment during seizures. Furthermore, it is possible that the threshold and timing of Fos activation varies between cell types, such that not all neurons recruited during seizures exhibit increased Fos labeling. In our study, mice in the seizing group exhibited different seizure frequencies and post-seizure intervals which could potentially lead to variability in the degree or extent of Fos activation.

In summary, our work uses Fos expression patterns to provide the first map of brain regions recruited by seizures in *Kcna1* -null mice. Our finding that spontaneous seizures in KO mice significantly activate Fos expression in extrahippocampal networks, namely the amygdala, suggests critical involvement of these limbic circuits in epilepsy in *Kcna1*- null mice.

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Abbreviations used:

KO	knockout
PBS	phosphate-buffered saline

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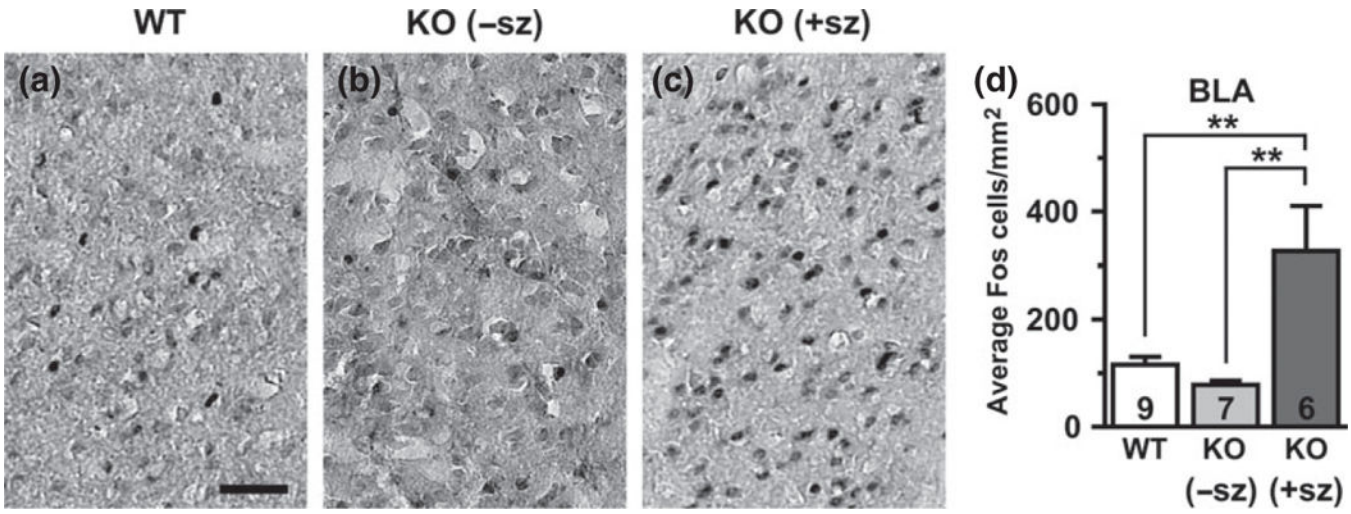

Fig. 1.

Fos expression in the basolateral amygdala (BLA) of a wild-type mouse (a), a non-seizing knockout (KO) mouse (b), and a seizing KO mouse (c). (d) Quantification of Fos-positive cells by group. Numbers in bars indicate number of animals analyzed. −sz, non-seizing; +sz, seizing. Scale bar: 50 μm, $^{**}p < 0.01$.

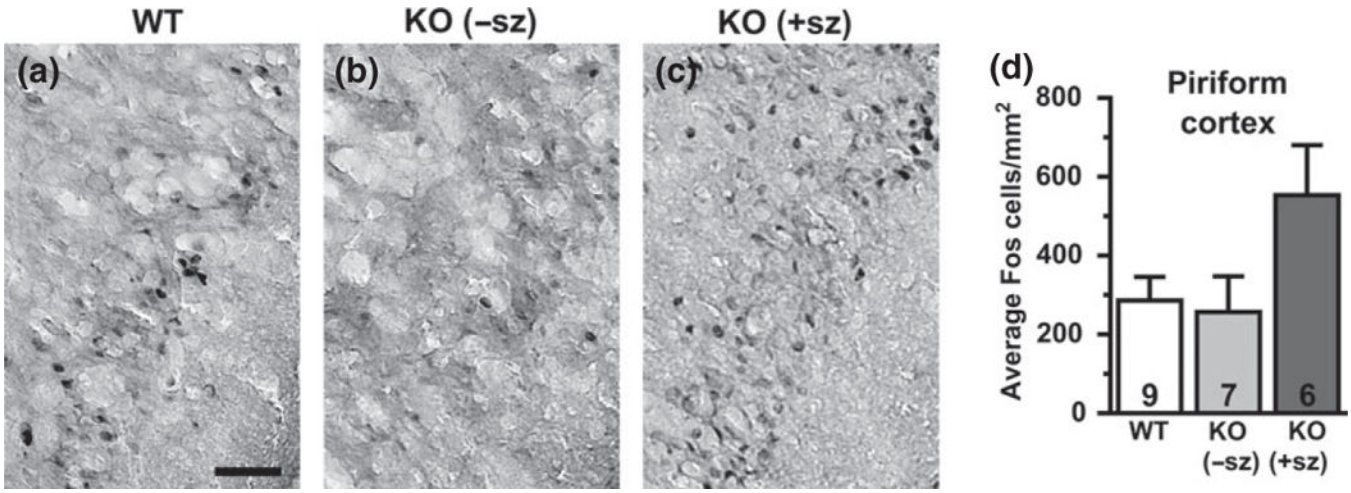

Fig. 2.

Fos expression in the piriform cortex of a wild-type mouse (a), a non-seizing knockout (KO) mouse (b), and a seizing KO mouse (c). (d) Quantification of Fos-positive cells by group. Numbers in bars indicate number of animals analyzed. −sz, nonseizing; +sz, seizing. Scale bar: 50 μm

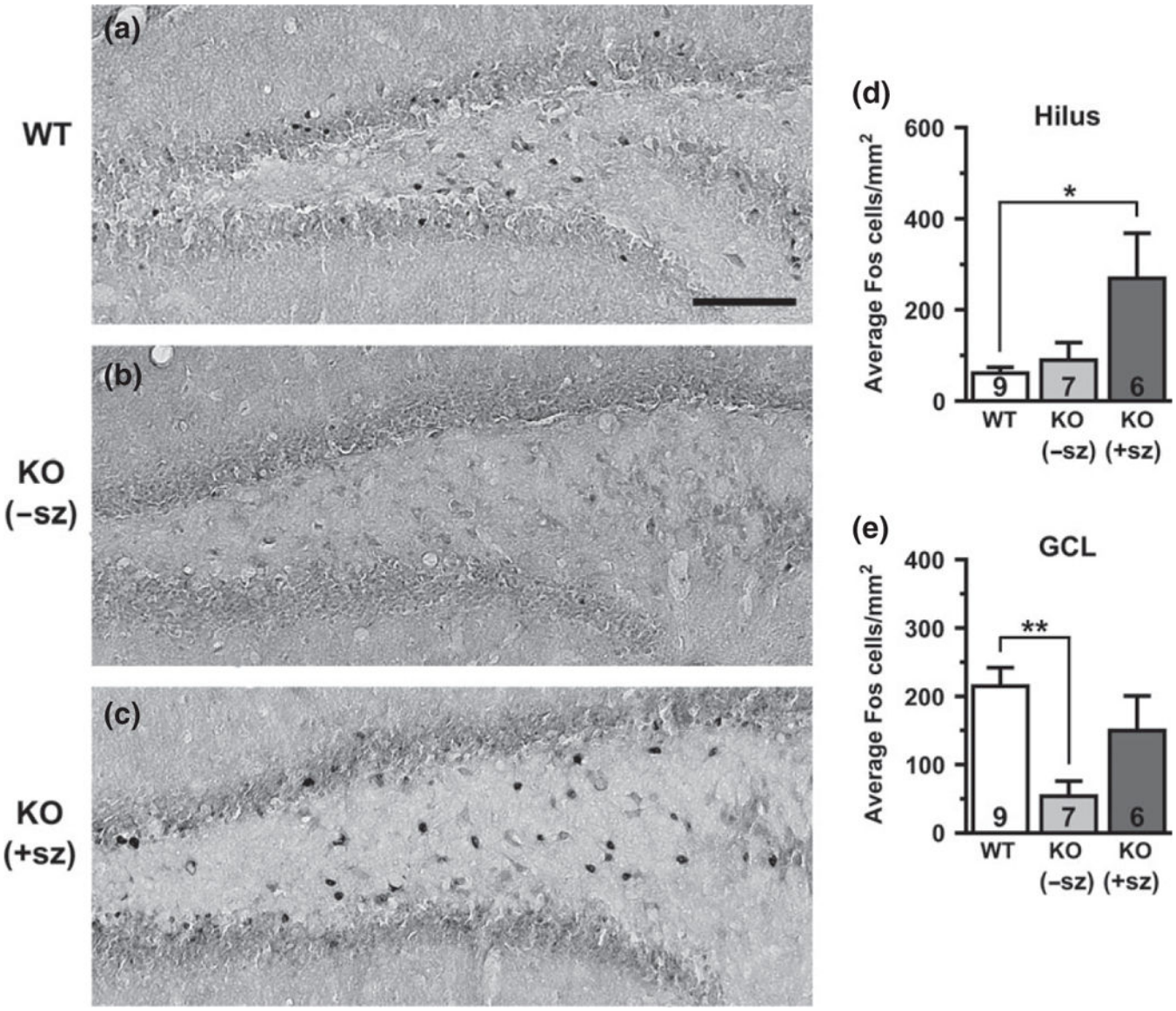

Fig. 3.

Fos expression in the dentate gyrus of a wild-type mouse (a), a non-seizing knockout (KO) mouse (b), and a seizing KO mouse (c). Quantification of Fos-positive cells by group in the hilus (d) and the granule cell layer (GCL) (e). Numbers in bars indicate number of animals analyzed. −sz, non-seizing; +sz, seizing. Scale bar: 100 μm. $*p < 0.05$; $**p < 0.01$.

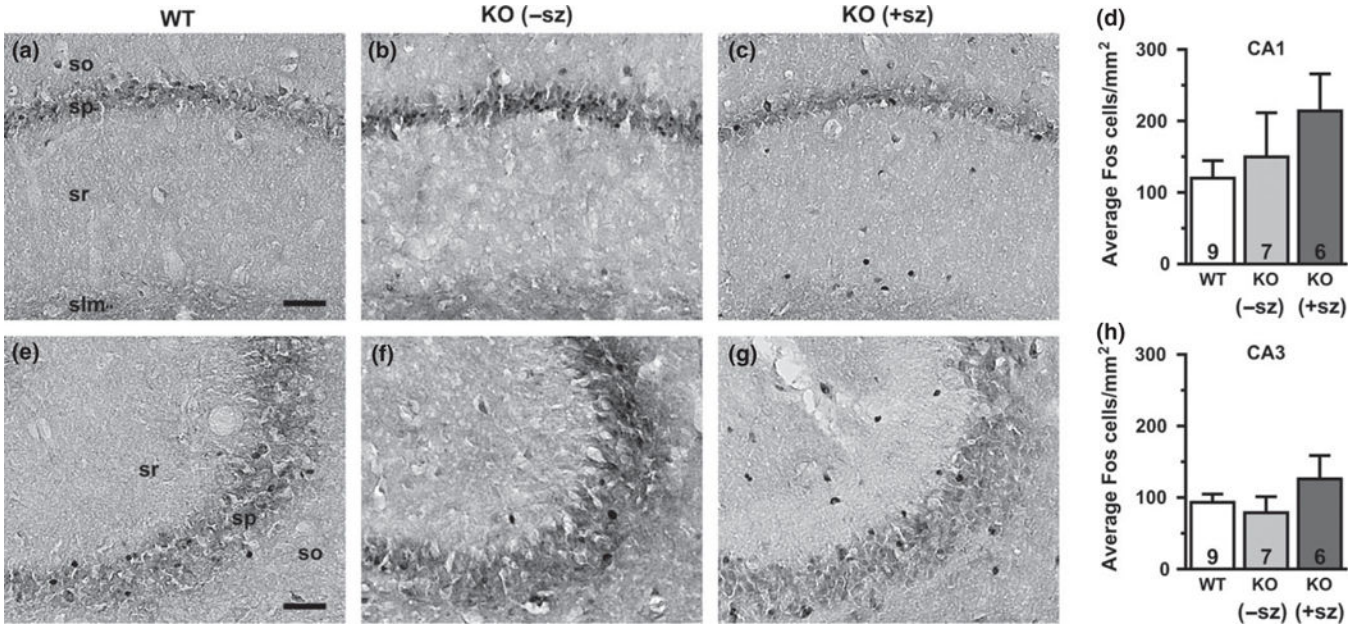

Fig. 4.

Fos expression in the hippocampal CA1 pyramidal cell layer of a wild-type mouse (a), a non-seizing knockout (KO) mouse (b), and a seizing KO mouse (c). (d) Quantification of Fos-positive cells by group in the CA1 pyramidal cell layer. Fos expression in the hippocampal CA3 pyramidal cell layer of a wild-type mouse (e), a non-seizing KO mouse (f), and a seizing KO mouse (g). (h) Quantification of Fospositive cells by group in the CA3 pyramidal cell layer. Numbers in bars indicate number of animals analyzed. −sz, non-seizing; +sz, seizing; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; slm, stratum lacunosum-moleculare. Scale bar: 50 μm.

Table 1

Behavioral seizures in Kcnal-null mice during the 4 h immediately before tissue harvesting

			Individual seizure characteristics		
Mouse number	**Age (d)**	**Total number of seizures**	**Approx. time between seizure onset and tissue harvesting (min)**	**Seizure duration (s)**	**Racine score**
1	80	3	42	27	4
			120	43	4
			240	34	3
2	79	4	143	60	4
			148	31	3
			159	41	3
			234	41	3
3	67	5	28	33	3
			67	27	3
			108	25	3
			145	34	3
			193	27	4
4	36	2	89	60	3
			167	60	3
5	34	2	40	38	5
			213	37	5
6	30	2	73	33	5
			91	31	5