

Neural Disruptions and Autism Spectrum Disorder Phenotypes: A Synapsin II Knockout Study

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Abstract

Autism spectrum disorders (ASD) and epilepsy are neurodevelopmental conditions that appear with high rate of cooccurrence, suggesting the possibility of a common genetic basis. Mutations in Synapsin (SYN) genes, particularly SYN1 and SYN2, have been recently associated with ASD and epilepsy in humans. Accordingly, mice lacking Syn1 or Syn2, but not Syn3, experience epileptic seizures and display autistic-like traits that precede the onset of seizures. Here, we analyzed social behavior and ultrasonic vocalizations emitted in 2 social contexts by SynI, SynII, or SynIII mutants and show that SynII mutants display the most severe ASD-like phenotype. We also show that the behavioral SynII phenotype correlates with a significant decrease in auditory and hippocampal functional connectivity as measured with resting state functional magnetic resonance imaging (rsfMRI). Taken together, our results reveal a permissive contribution of Syn2 to the expression of normal socio-communicative behavior, and suggest that Syn2-mediated synaptic dysfunction can lead to ASD-like behavior through dysregulation of cortical connectivity.

Key words: autism, functional connectivity, social behavior, synapsin, ultrasonic vocalizations

Introduction

Autism Spectrum Disorder (ASD) is a group of disorders including qualitative impairments in social interaction and communication, as well as aberrant repetitive behaviors, with symptoms emerging early in childhood ([American Psychiatric Association 2013](#)).

In association with ASD core symptoms, autistic subjects commonly exhibit other associated features generating a variety of comorbidity traits including epilepsy (with 30% of prevalence) ([Levy et al. 2010](#); [Fassio et al. 2011](#); [van Steensel et al. 2011](#); [Woolfenden et al. 2012](#); [Gilby and O'Brien 2013](#)). Conversely, ASD

traits are often present in children with epilepsy (with 46% of prevalence) ([Clarke et al. 2005](#); [Matsuo et al. 2010](#)). Given the high comorbidity between ASD and epilepsy, the possibility of common genetic bases and pathogenic mechanisms for both diseases have been proposed ([Jeste and Tuchman 2015](#)).

Evidence of altered brain structural organization and development has been provided by recent structural magnetic resonance imaging (MRI) studies in ASD and epilepsy. This includes examples of abnormal brain growth during the first year of life and regional differences in the cerebral cortex, limbic system, and cerebellum ([Courchesne et al. 2004](#); [Schumann et al. 2004](#); [Allen et al. 2005](#); [Carper and Courchesne 2005](#); [Santos et al. 2011](#)). From a functional standpoint, it has been proposed that both epilepsy and ASD may entail atypical connectivity, or impaired communication between regions, with imaging studies showing a prevalent contribution of reduced long-range functional synchronization, at least in the adult brain ([Waites et al. 2006](#); [Pittau et al. 2012](#); [Di Martino et al. 2013](#); [Holmes et al. 2013](#); [Uddin et al. 2013](#); [Haneef et al. 2014](#); [He et al. 2015](#); [Vasa et al. 2016](#)). Similar findings have been recently recapitulated in autism mouse models ([Sforazzini et al. 2014, 2016](#); [Liska et al. 2017](#)). These results point at an involvement of alterations in synaptic connectivity and macroscale brain communication in the pathogenesis and the manifestations of ASD. However, the causal role of specific genetic etiologies in the reorganization of resting state networks remains elusive.

Recently, mutations in Synapsin (SYN) genes and particularly in both SYN1 and SYN2 have been described in subjects with ASD and/or epilepsy ([Lakhan et al. 2010](#); [Fassio et al. 2011](#); [Corradi et al. 2014](#); [Nguyen et al. 2015](#)). Syns are a family of neuronal phosphoproteins, encoded by 3 distinct genes (SYN1, SYN2, and SYN3) that regulate synaptic transmission and plasticity, as well as neuronal development ([Cesca et al. 2010](#); [Fornasiero et al. 2010](#); [Giovedì et al. 2014](#)). The principal functions of Syns are to control synaptic

vesicle (SV) trafficking and modulate neurotransmitter release at the presynaptic terminal. Syn isoforms seem to play distinct roles in excitatory and inhibitory neurons and their deletion causes an imbalance between excitation and inhibition, which is reported as one of the possible causes underlying autism and epilepsy ([Gitler et al. 2004](#); [Baldelli et al. 2007](#); [Chiappalone et al. 2009](#); [Farisello et al. 2013](#); [Medrihan et al. 2015](#)).

SynI, SynII, double SynI/II and triple SynI/II/III knockout (KO) mice are prone to epileptic seizures starting from 2 to 3 months of age and their phenotype progressively aggravates with age. The fact that epilepsy does not appear at birth and that SynIII KO mice are not epileptic, can be explained by the specific expression profiles of the 3 Syn genes during development ([Ferreira et al. 2000](#); [Feng et al. 2002](#); [Bogen et al. 2009](#)). Syn1 and Syn2 are expressed at low levels at birth and their expression progressively increases along synaptogenesis to reach a stable plateau at 1–2 months of life, approximately the time window within which epilepsy appears. By contrast Syn3, encoding for an isoform mostly involved in the early phases of neural development, is expressed at very early developmental stages and is downregulated afterwards ([Bogen et al. 2009](#); [Porton et al. 2011](#); [Giovedì et al. 2014](#)).

Interestingly, Syn mutant mice display deficits in social behavior with SynII mutants showing the most severe phenotype ([Dyck et al. 2009, 2012](#); [Greco et al. 2013](#)). Recent electrophysiological and pharmacological studies also showed that deletion of Syn2 is associated with a specific loss of asynchronous GABA release at inhibitory synapses, causing a hyperexcitability profile in mice due to the substantial lack of the GABA tonic current ([Medrihan et al. 2013, 2015](#)). In keeping with this, it has recently been shown that THIP, a selective agonist of δ subunit-containing extrasynaptic GABA_A receptors, is effective in reverting the epileptic phenotype of SynII KO mice both *ex vivo* and *in vivo* ([Medrihan et al. 2015](#)), consistent with a role of the decreased GABAergic tone in epileptogenesis.

Here we examined socio-communicative behavior in mouse mutants lacking Syn1, Syn2, or Syn3 to unravel the contribution of these genes as risk factors for the development of ASD and neurodevelopmental disorders. We show that mice lacking Syn2 exhibit a robust ASD-like behavioral phenotype, a finding associated with reduced functional connectivity in auditory cortices and hippocampal regions. We also show that pharmacological treatments that revert epileptic phenotype do not improve autistic-like behaviors in SynII KO mice, suggesting distinct pathomechanisms for epilepsy and autism-like

phenotypes in these mice. Collectively, these findings reveal a permissive contribution of *Syn2* to the expression of normal socio-communicative behavior, and suggest that *Syn2*-mediated synaptic dysfunction can lead to ASD-like behavior through dysregulation of cortical connectivity.

Materials and Methods

Animals

SynI, *SynII*, and *SynIII* mutant mice (Chi et al. 2001; Feng et al. 2002; Gitler et al. 2004) were backcrossed to a C57BL/6 J background (Charles River, Calco, Italy) for more than 10 generations. HET male X HET female mice were mated in the *SynII* and *SynIII* mutant lines. Given the localization of the *SynI* gene on the X chromosome, HET females were bred with WT males and only male WT and KO pups were tested. All procedures involving animals and their care were carried out in accordance with the guidelines established by the European Community Council (Directive 2014/26 EU) and were approved by the Italian Ministry of Health.

Behavioral Experiments

Maternal Separation Test

At postnatal day (pnd) 4, 6, 8, and 12, *SynI* male and *SynII* and *SynIII* male and female pups were individually subjected to a 3-min maternal separation test. Ultrasonic vocalizations (USVs) and spontaneous behaviors were recorded and analyzed as in Scattoni et al. (2008) and Romano et al. (2013). After each recording session, body weight, body temperature, and righting reflex were measured as in Scattoni et al. (2008) and Romano et al. (2013).

Adult Male–Female Social Interaction Test

At 3 months of age, all male mice of the 3 genotypes were tested in a 3-min male–female social interaction test. USVs and social behaviors were recorded and analyzed as in Scattoni et al. (2011) and Michetti et al. (2014). A second group of *SynII* WT and KO male mice was subjected to the same male–female social interaction test after a 1-week treatment with the selective agonist of δ -subunit-containing GABA_A receptor (THIP). ALZET-1002 osmotic minipumps, filled with THIP (2.5 mg/kg) or artificial cerebrospinal fluid (ACSF) were implanted subcutaneously to achieve a 1-week continuous treatment (Medrihan et al. 2015).

Statistical Analysis

A mixed-model ANOVA with Repeated Measures was performed to analyze genotype-dependent effects on neonatal USVs and spontaneous movements in the postnatal phase.

Univariate ANOVAs were conducted for behaviors and USVs analyzed during the male–female social interaction test. The probability of vocalizations within genotype was calculated as number of calls in each category per subject/total number of calls and standardized by angular transformation. Because no sex differences in calling patterns and spontaneous movements were detected in pups, data were collapsed across sex. Data are expressed as mean \pm SEM with a significance level of $P < 0.05$. Post hoc comparisons were performed using Tukey's test only when a significant F-value was determined.

Structural and Functional MRI

Voxel-Based Morphometry

Morpho-anatomical MRI was carried out on paraformaldehyde-fixed specimens obtained from the same WT and *SynI*, *SynII*, or *SynIII* KO mice previously subjected to behavioral experiments (for details on fixation procedure see Dodero et al. 2013). Brains were imaged inside intact skulls to avoid post-extraction deformations. MR images were acquired at 7.0 Tesla with a 3D fast spin-echo sequence, TR = 550 ms, TE = 33 ms, RARE factor = 8, echo spacing 11 ms, and voxel size of 90 μ m (isotropic). Intergroup differences in local gray

matter (GM) volume were mapped with Voxel-Based Morphometry (VBM) (Ashburner and Friston 2000; Pagani et al. 2016a). Volumes of cortical and subcortical regions were calculated via automated anatomical labeling (Janke et al., 2012; Pagani et al. 2016b).

Resting State Functional MRI (rsfMRI)

Resting state functional MRI (rsfMRI) experiments were performed on adult male *Syn II* mice (N = 10 WT, N = 10 KO) previously used for THIP experiments (VEH animals) under shallow halothane anesthesia (0.75%). The animal preparation protocol was recently described in great detail (Ferrari et al. 2012; Sforazzini et al. 2016). Single-shot BOLD rsfMRI time series were acquired using an echo planar imaging sequence with TR/TE 1200/15 ms, flip angle 30°, matrix 100 \times 100, field of view 2 \times 2 cm², 24 coronal slices, slice thickness 0.50 mm and 500 volumes. rsfMRI images were preprocessed as previously described in great detail (Liska et al. 2017). To obtain an unbiased identification of the brain regions exhibiting genotypedependent differences in functional connectivity, we employed the global brain connectivity method (Cole et al. 2011; Liska et al. 2015). This metric considers connectivity of a given voxel to all other voxels simultaneously by computing average connectivity strength. We also computed local connectivity strength by limiting this measurement to connections within a 0.6252 mm sphere around each voxel (6 voxels in plane), while long-range connectivity was computed by considering only connections to voxels outside this sphere. The effect was also quantified in volumes of interest (VOIs). The anatomical location of the examined VOIs is reported in Supplementary Figure S6. Intergroup differences in the antero-posterior extension and intensity of long-range rsfMRI correlation of auditory regions were mapped using a seed-based approach (Sforazzini et al. 2014). Bilateral seed regions (3 \times 3 \times 1 voxels) were chosen to cover foci exhibiting significant reductions in local connectivity. Seed locations are reported in Figure 5 and Supplementary Figure S7 (red dots). Group level differences in connectivity distributions were mapped using 2-sample Student's t-tests ($t(19) = 2.3$, cluster corrected with $p < 0.05$, as implemented in FMRIB Software Library). Alterations in inter-hemispheric functional connectivity were assessed by computing correlation coefficients of inter-hemispheric VOIs covering cortical regions. The statistical significance of intergroup correlation strength in each region was assessed with a Student's t-test, followed by multiple comparison correction (false discovery rate, $q = 0.05$ according to the Benjamini–Hochberg procedure).

Results

Social Communication Deficits in *SynI* Mice

SynI KO pups separated from their mothers and siblings emitted significantly less calls and for shorter times than their WT littermates, even if both genotypes showed a peak of emission at pnd 8 (Fig. 1A, D). Moreover, an overall genotype effect was also found in USV characteristics showing a decrease in peak frequency and peak amplitude (Supplementary Fig. S1A, B). However, no major alterations were found in the production of USVs by call categories (Supplementary Fig. S2A), or in motor development, somatic growth and body temperature (data not shown). In line with neonatal USVs, a robust deficit in USVs emission rate was detected in adult *SynI* KO mice during courtship. Analysis of the social investigation (total amount of sniffing responses) and social communication in adult *SynI* mice interacting with an estrus female revealed that KO mice spent less time and less frequently investigated the C57BL/6J female partner than WT mice (Fig. 2A, D), and, similarly, vocalized less (Fig. 2G). Moreover, the USVs emitted by KO animals were characterized by a shorter duration and a decreased peak frequency (Supplementary Fig. S3A, D). Despite KO mice emitted a very low number of vocalizations in this social context, their vocal repertoire was still intact as compared to WT (Supplementary Fig. S4A, D).

Complete ASD-like Phenotype in SynII Mice

SynII mice exhibited by far the most robust ASD-like phenotype. During the maternal separation test, the total number of vocalizations varied across genotype, and in particular after pnd 6, KO pups rapidly decreased their calling rate (Fig. 1B). A main effect of genotype was also found in the total calling time albeit not in a gene-dosage dependent manner. Indeed, post hoc comparisons revealed that KO pups vocalized less than HET but their calling time was comparable to WT mice ($P < 0.05$) (Fig. 1E). Moreover, USV data were characterized by an overall genotype effect in mean duration (Supplementary Fig. S1C), while no differences were found in peak amplitude and peak frequency (data not shown). No genotype differences were observed in the SynII vocal repertoire (Supplementary Fig. S2B), suggesting that, as for SynI, the observed alterations did not affect qualitative characteristics of the vocalizations.

Interestingly, several deviations from normative motor development were also observed in SynII KO pups during the first 12 pnds. In particular, SynII KO pups spent more time to right their body than WT and HET at pnd 6 as confirmed by post hoc analyses (Fig. 3A). We did not find genotype differences in somatic growth or body temperature (data not shown), while, when we measured spontaneous behavior, an overall genotype effect was found for pivoting frequency and circling frequency and duration (Fig. 3B, D).

Male–female social interaction test in adulthood confirmed the presence of ASD-like behaviors in SynII KO mice. Male KO mice interacting with a receptive female displayed reduced levels of social sniffing behavior associated with a complete lack of social communication (Fig. 2B, E, H and Supplementary Movie). A main effect of genotype was also observed for the mean duration of USVs, with SynII KO mice vocalizing with

confirming previous reports of increased repetitive behaviors in this mouse line (Greco et al. 2013).

Absence of Autistic-like Traits in SynIII Mice

No genotype differences were observed in SynIII mutants for all parameters analyzed during maternal separation test (Fig. 1C, F and Supplementary Fig. S2C), including somatic growth and body temperature (data not shown), as well as adult social interaction (Fig. 2C, F, I and Supplementary Figs S3C and 4C, F).

Largely Typical Brain Volume and Morphometry in SynI, SynII, and SynIII KO Mice

Whole-brain VBM of GM did not reveal any focal or generalized inter-strain differences for each of the 3 genotypes ($Z > 1.6$, cluster correction $P = 0.05$). In keeping with this, the 3dimensional volumetric assessment of cortical and subcortical structures did not reveal any significant intergroup differences (Supplementary Fig. S5). These results argue against a contribution of major GM neuroanatomical alterations to the observed behavioral phenotypes.

Synapsin II KO Mice Show Abnormal Functional Connectivity

Because SynII KO mice showed the most severe ASD-like phenotype, and given the established observation of network disruption in autism (Liska and Gozzi 2016) we decided to perform a functional connectivity analysis on these mice. Specifically, to identify macroscale functional substrates involved in the

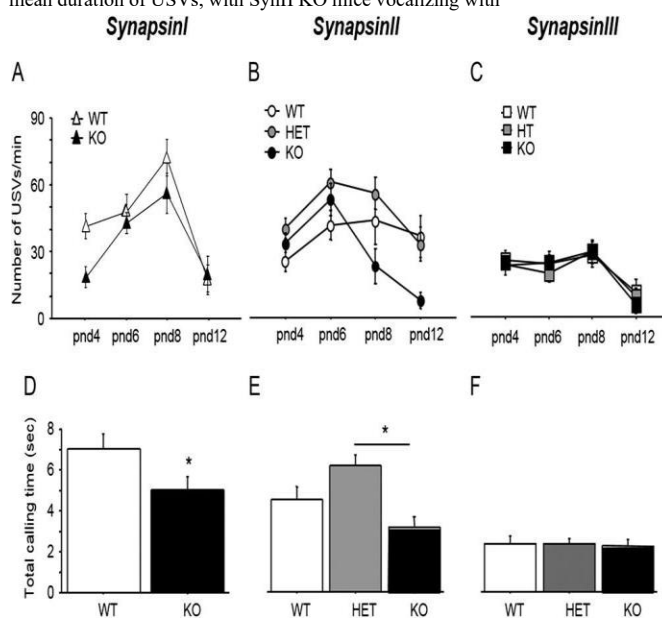


Figure 1. Vocalizations emitted by pups of the 3 mutant lines SynI, SynII, and SynIII in response to a 3-min maternal separation. (A–C) Number of vocalizations on postnatal days (pnd) 4 through 12. Total number of vocalizations varied across genotypes in SynI [genotype effect: $F_{1,30} = 4.561$, $P = 0.041$] and SynII pups [genotype effect: $F_{2,70} = 3.424$, $P = 0.038$]. No genotype differences were found for SynIII pups. (D–F) Total time spent to vocalize indicated as total calling time (total number of vocalizations \times mean duration). Data pooled from all ages considered. A main effect of genotype was found in total calling time in SynI [genotype effect: $F_{1,30} = 4.060$, $P = 0.05$] and SynII mouse pups [$F_{2,70} = 4.153$, $P = 0.01$]. No genotype differences were found for SynIII pups. For all graphs data are expressed as means \pm SEM * $P < 0.05$. SynI: $N = 15$ WT and 17 KO; SynII: $N = 21$ WT, 40 HET and 12 KO; SynIII: $N = 16$ WT, 32 HET and 23 KO.

shorter calls than WT and HET mice (Supplementary Fig. S3B). The classification of the vocalizations into distinct categories allowed the detection of limited vocal repertoire in adult KO males as compared to WT and HET littermates (Fig. 2J and Supplementary Fig. S4B, E). KO mice emitted a low number of complex, 2-components, upward, and frequency steps calls, and limited their vocalizations to short calls (54%).

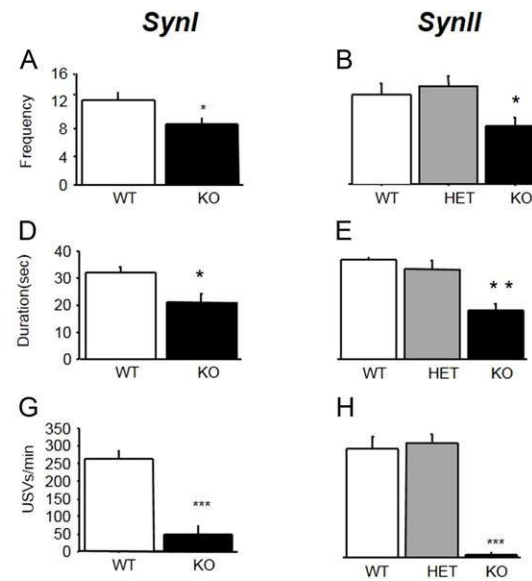
Interestingly, during this test, a main effect of genotype was found for grooming frequency and duration (Fig. 2K). Adult KO mice performed self-grooming more frequently and for longer time than WT and HET mice,

behavioral alterations of SynII KO, we mapped local and long-range connectivity in these mutants using rsfMRI as previously described (Liska et al. 2015). No significant genotype-dependent effects in global connectivity strength were observed. However, local (short range, <0.6 mm) connectivity mapping revealed bilateral foci of decreased local connectivity in auditory and somatosensory cortices and intermediate hippocampus in SynII KO mice as compared to WT littermates (Fig. 4A). Interestingly, local connectivity in auditory areas and intermediate hippocampus were significantly correlated with USV calls impairment in individual subjects (Fig. 4B). Inter-hemispheric connectivity between the auditory cortex and its homotopic contralateral

regions was also significantly reduced (Supplementary Fig. S6). No impairments in inter-hemispheric connectivity were observed within other cortical regions (Supplementary Fig. S7). Seed-based mapping of auditory regions also revealed decreased connectivity between these areas and prefrontal and cingulate afferents in SynII mutants (Fig. 5), suggesting the presence of long-range fronto-parietal desynchronization in these mice.

Rescue of Epilepsy in SynII KO Mice does not Affect ASD-like Phenotypes

SynII KO mice are characterized by a sharp decrease in asynchronous GABA release and tonic inhibition in the hippocampus, and correction of tonic inhibition with the extrasynaptic GABA_A receptor agonist THIP rescues their epileptic phenotype



Additional deficits in

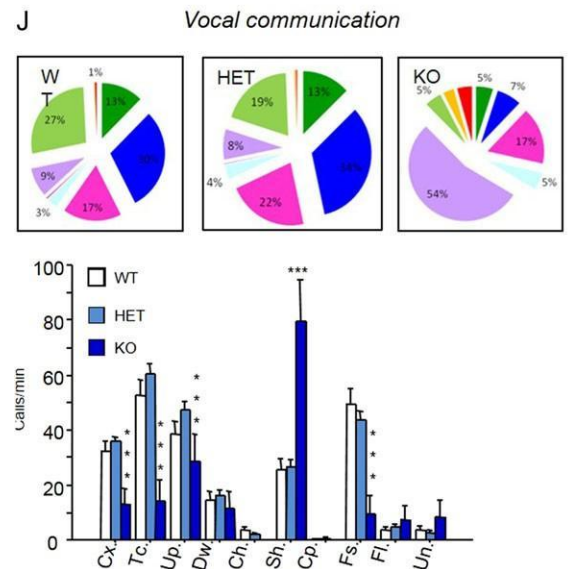


Figure 2. Adult male–female social interaction test in SynI, SynII, and SynIII male mice. (A–C) Number of sniffing of C57BL/6 J female. SynI KO and SynII KO mice sniffed the C57BL/6 J female partner less frequently than WT mice ($F_{2,39} = 8.34, P = 0.006$; genotype effect in SynII: $F_{2,39} = 3.71, P = 0.03$), as confirmed by post hoc comparisons. (D–F) Duration of sniffing shorter time than WT mice (and HET mice in the SynII mutant line) [genotype effect in SynI: $F_{1,30} = 8.34, P = 0.007$; genotype effect in SynII: $F_{2,39} = 7.95, P < 0.001$]. (G–I) Mean number of USVs emitted by male mice in this context. SynI KO mice emitted a lower number of vocalizations than WT mice (and HET mice in the SynII mutant line) [genotype effect in SynI: $F_{1,30} = 8.34, P = 0.007$; genotype effect in SynII: $F_{2,39} = 7.95, P < 0.001$]. The values represent the percentages of the different call categories within each genotype. The bar plot represents the percentage of each call category within the total calls for the subject. SynII KO emitted a completely different vocal repertoire probably due to the absence of vocalizations with respect to their WT and HET littermates [genotype effect: $F_{1,30} = 8.34, P = 0.007$; genotype effect in SynII: $F_{2,39} = 7.95, P < 0.001$]. Data are expressed as means \pm SEM. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. and 10 KO; SynIII: N = 9 WT, 13 HET, 9 KO.

(Medrihan et al. 2013, 2015). To investigate if the abnormal inhibitory neurotransmission causing epilepsy in SynII KO mice is also responsible for the observed ASD-like behaviors, we evaluated whether treatment with THIP was also able to ameliorate the behavioral deficits identified during a male–female social interaction test. Interestingly, the analysis of social investigation, number of vocalizations and self-grooming did not detect a significant effect of THIP treatment (Fig. 6A–C). The inability of THIP to revert the social behavior and communication deficits of SynII KO mice, while rescuing their epileptic

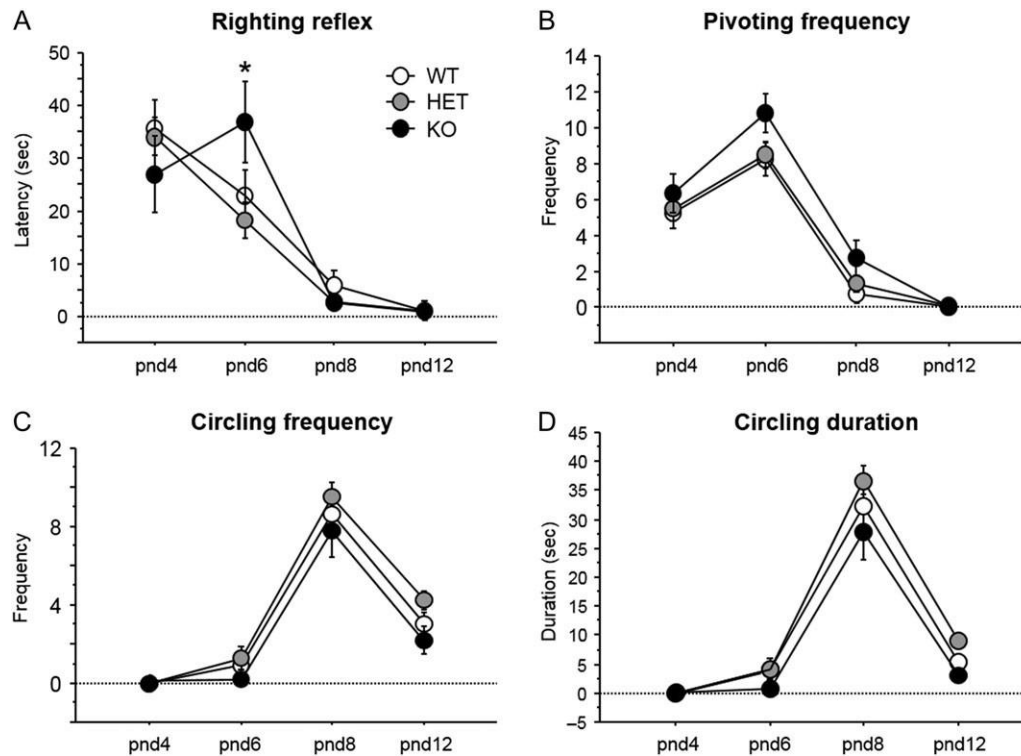


Figure 3. Spontaneous behaviors and righting reflex in SynII mouse pups during the first 12 pnd. Frequency and duration of behavioral patterns showed by SynII pups during 3 min of maternal separation: (A) Righting reflex latencies measured after the recording sessions. Post hoc comparisons indicated that SynII KO spent more time to right their body than SynII WT and HET at pnd 6 [$F_{2,210} = 2.436$; $P < 0.05$]. (B) A main effect of genotype was found for pivoting frequency [$F_{2,70} = 3.19$; $P < 0.05$]. (C, D) Circling frequency and duration. Analysis for both parameters reported a significant main effect of genotype [frequency: $F_{2,70} = 3.50$; $P < 0.05$ and duration: $F_{2,70} = 3.33$; $P < 0.05$]. For all graphs data are expressed as mean \pm SEM, * $P < 0.05$. $N = 21$ WT, 40 HET and 12 KO. SynII KO mice showed social communication and interaction deficits both in postnatal and adult phases. This social deficit was not associated with genotype differences in the use of specific call categories (vocal repertoire), suggesting that the impairment in SynI is limited to the calling rate. Overall, the lack of SynI mainly affects social communication throughout the lifespan. SynII KO pups emitted a low number of vocalizations after pnd 6, and showed several deviations from normative motor development during the first 12 pnds. The delayed maturation of the motor behavior is in line with the transient delay in sensory-motor development shown by other animal models of ASD such as *Mecp2*-null, *NL3*, and *Reeler* mice (Picker et al. 2006; Chadman et al. 2008; De Filippis et al. 2010; Romano et al. 2013). The early motor abnormalities found in KO pups are particularly intriguing in view of the subtle alterations in infantile reflexes (such as body righting or head tilting) and general movements described in infants later diagnosed for autism (Teitelbaum et al. 2004; Phagava et al. 2008; Esposito et al. 2011). In adulthood, male SynII KO mice displayed low levels of social sniffing behavior together with a complete lack of vocal communication while interacting with a receptive female, showing a most severe deficit respect to SynI mice. These alterations are unlikely to be due to olfactory impairments, as previous research revealed conserved olfactory reactivity in these mice (Greco et al. 2013). Interestingly, during this test, SynII KO mice displayed also high levels of self-grooming

Discussion

ASD frequently occurs together with epilepsy, suggesting common etiology and pathogenic mechanisms (Jeste and Tuchman 2015). An array of mutations in *SYN1* and *SYN2* genes have been recently associated with both disorders in humans (Cesca et al. 2010; Fomasiero et al. 2010; Fassio et al. 2011; Corradi et al. 2014). The mechanisms by which deletion of *SYN1* and *SYN2* leads to epilepsy propensity has been recently clarified in *Syn* mutant mice and found to involve specific impairments of phasic and tonic GABAergic transmission, resulting in an excitation/inhibition imbalance at synapses and an increased excitability of excitatory neurons (Gitler et al. 2004; Baldelli et al. 2007; Chiappalone et al. 2009; Farisello et al. 2013; Medrihan et al. 2013, 2015). *Syn* mutant mice also display alterations in social behavior (Dyck et al. 2012; Greco et al. 2013), but the pathogenic mechanisms that link *SYN* mutations to ASD, as well as the emergence of the core symptoms of ASD, have not been clarified yet.

Social Deficits in SynI and a Complete ASD-like Phenotype in SynII KO Mice

Previous behavioral characterization of *SynI*, *II*, and *III* mice revealed that *SynII* mutants exhibit the most pronounced set of ASD-like alteration (Greco et al. 2013). In this study, we carried out a detailed investigation of the socio-communicative deficits in these mice, the first ASD core symptom. To this aim, we investigated neonatal and adult vocal, and social repertoire in the 3 *Syn* mutant mouse lines. Our results show largely distinct phenotypes in the 3 mutant lines, with the most severe ASDlike phenotype in *SynII* KO mice.

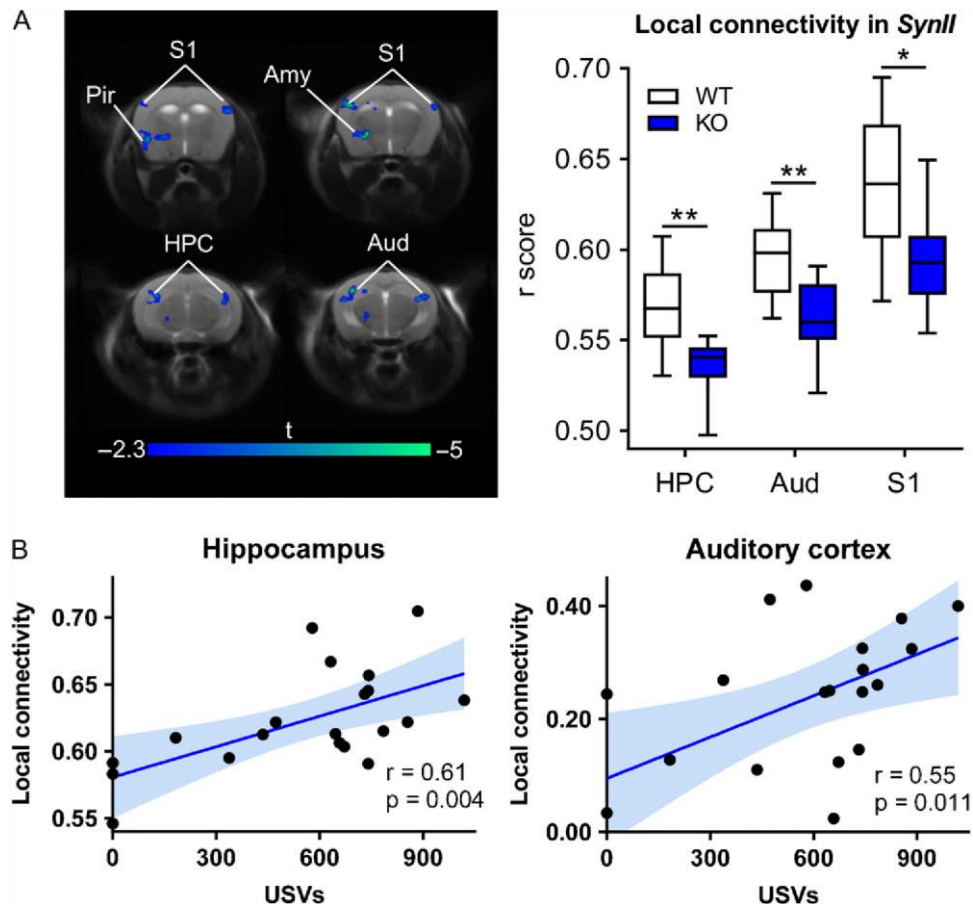


Figure 4. *SynII* KO mice show reduced local functional connectivity. (A) Functional connectivity mapping revealed a bilateral decrease in rsfMRI local connectivity in primary auditory and somatosensory cortices as well as in hippocampal regions of *SynII* KO mice compared to control littermates ($t < -2.3$, $p < 0.001$). These effects were also quantified in VOIs, confirming the presence of significantly reduced local connectivity in these regions. Focal reduction in local rsfMRI connectivity was also found in the left amygdala and piriform cortex of *SynII* mice. (B) Local rsfMRI connectivity in the auditory cortex and hippocampus were associated with lower number of USVs, suggesting a possible involvement of these brain regions in the emergence of the ASD-related behavior in *SynII* mice. * $P < 0.05$, ** $P < 0.01$. Amy, amygdala; Aud, auditory cortex; HPC, hippocampus; Pir, piriform cortex; S1, somatosensory cortex.

behavior, corroborating the presence of repetitive behaviors in this mutant line (Greco et al. 2013), and confirming the presence of a full ASD-like phenotype in this mutant line.

By contrast, *SynIII* KO mice did not display epileptic or ASD-like behaviors. This finding has 3 possible mechanistic implications: 1) *Syn* isoforms exert distinct functional roles in brain development and function; 2) only "adult" *Syn* isoforms (i.e., *SynI* and *SynII*) are necessary for the correct formation, maintenance and plasticity of synapses that contribute to short- and long-range synaptic connectivity within and across brain regions; and 3) as *SynIII* is expressed earlier than *SynI* and *SynII* during brain development and is downregulated afterwards, the data identify a window of vulnerability for ASD in the perinatal period of synapse formation and rearrangement, when most of synaptic genes start to be potently expressed.

The ASD-like Phenotype of *SynII* KO is Correlated with an Impaired Functional Connectivity

MRI studies have reported structural and connectivity brain alterations in ASD and epileptic patients. We therefore tested whether an altered structural/functional connectivity underlies the unusual behavioral patterns of *Syn* mice. The absence of major brain structure alterations observed in our MRI studies in either mutant strain rules out a role of these genes in shaping macroscale brain anatomical organization. However, the presence of reduced functional connectivity in auditory regions and long-range hypoconnectivity between this region and cingulate prefrontal areas in *SynII* KO mice, was significantly correlated with the USV phenotype.

This observation is consistent with a large body of evidence pointing at an altered functional connectivity in ASD (Ecker and Murphy 2014), and corroborates functional imaging data obtained in other mouse models of ASD, in which a similar hypoconnectivity has been recently described (Dodero et al. 2013; Sforzini et al. 2016; Liska et al. 2017). The observation of an association between reduced parietal connectivity and impaired social communications supports the neurobiological relevance of these findings, and suggest that loss-of-function *SYN2* mutations can predispose to neurodevelopmental disorders and ASD through regional dysregulation of local and long-range functional network coupling.

The Epileptic and ASD-like Phenotypes of *SynII* KO Mice do not Share the Same Mechanism

Given the significant comorbidity of epilepsy and ASD, it has been suggested that common molecular alterations,

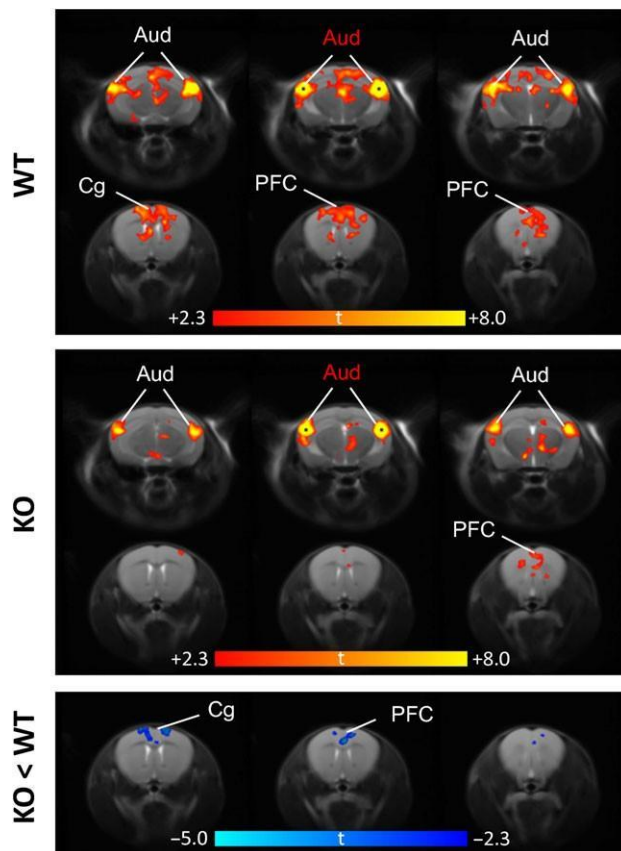


Figure 5. SynII KO mice show reduced fronto-temporal functional connectivity. Representative coronal slices depicting rsfMRI correlation maps ($t > |2.3|$, $p < 0.001$) with respect to bilateral auditory seed regions (black dots) in WT (top), and SynII KO mice (middle), plus genotype-dependent difference related to this connectivity patterns (bottom). Note the presence of decreased connectivity between auditory the prefrontal/anterior cingulate cortex observed in SynII KO mice. Slices have been arranged in a caudorostral manner (left to right, and top to bottom) and red labeling indicates seed location. AudI, primary auditory cortex; Cg, cingulate cortex; PFC, prefrontal cortex.

impinging on the synapse, may promote both pathological states. Given the strong ASD-like phenotype observed in epilepsy-prone SynII KO mice, we tested whether a treatment, that previously proved effective in fully rescuing the epileptic phenotype in these mice (Medrihan et al. 2015) and in a mouse model of fragile X syndrome (Olmos-Serrano et al. 2011), could also ameliorate the ASD-like phenotype. Importantly, we found that the treatment did not improve the social deficits and repetitive behaviors in SynII KO mice. This finding suggests that alterations in GABA-mediated tonic inhibition exhibited by this mutant line are not entirely responsible for the observed ASD-like behavioral deficits. This negative result implies that, while epilepsy may be the direct consequence of an excitatory/inhibitor imbalance, ASD-like symptoms are more likely to depend on abnormal functional connectivity and impaired adaptation to external stimuli. More broadly, this finding suggests that epilepsy and ASD follow distinct pathogenic pathways in spite of their largely shared genetic bases. The finding is also consistent with the observation that anti-epileptic drug treatments trying to correct the excitation/ inhibition imbalance only marginally ameliorate the core symptoms of ASD (Hirota et al. 2014).

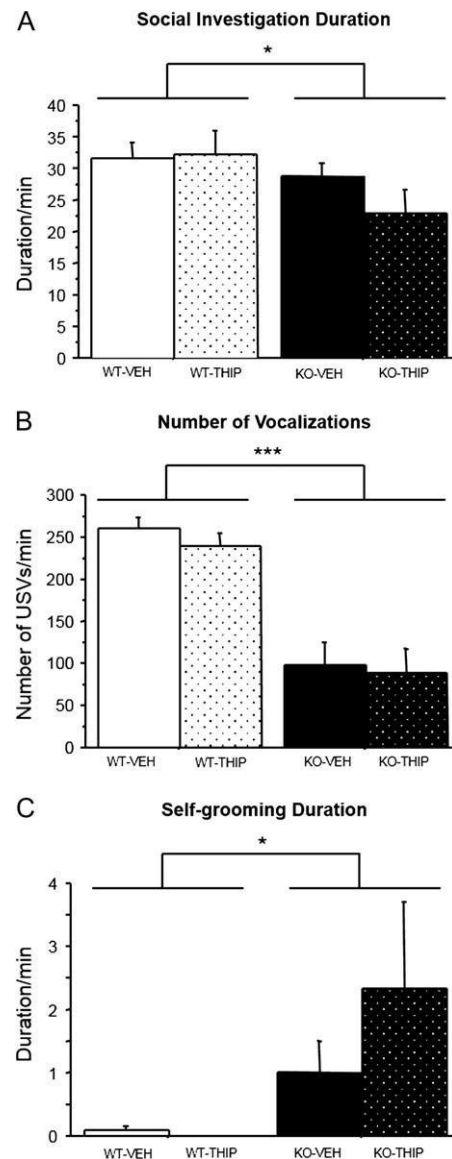


Figure 6. Effects of the extrasynaptic GABA_A receptor agonist THIP in SynII KO mice during a male-female social interaction test. Behavior and vocalizations emitted by WT and SynII KO male mice pretreated for 7 days with either vehicle or THIP while interacting with a receptive C57/BL6J female. (A) Mean duration of social investigation. (B) Mean number of USVs. (C) Total duration of self-grooming behavior. Analysis of the 3 parameters did not detect a significant effect of THIP. On the contrary, the genotype effect was maintained for all parameters [social investigation: $F_{1,43} = 4.026$, $P = 0.05$; number of USVs: $F_{1,43} = 49.503$, $P < 0.0001$, self-grooming duration: $F_{1,43} = 3.983$, $P = 0.05$]. Data are expressed as mean \pm SEM. * $P < 0.05$; *** $P < 0.0001$. $N = 11$ WT-vehicle, 11 WT-THIP; 12KO-vehicle; 12K-THIP.

Conclusions

Prior human and animal studies suggest that SYN genes represent a possible common genetic basis for ASD and epilepsy. Our behavioral characterizations in SynI and SynII KO mice support the view that these genes are involved in the expression of ASD-like behavioral traits, with Syn2 playing a crucial role in the establishment of both symptoms through a possible derangement of cortical connectivity, and identify these mutant lines as useful experimental models to address the pathogenesis of ASD and test new therapeutic strategies.

References

- Allen G, McColl R, Barnard H, Ringe WK, Fleckenstein J, Cullum CM. 2005. Magnetic resonance imaging of cerebellar-prefrontal and cerebellar-parietal functional connectivity. *Neuroimage*. 28:39–48.
- American Psychiatric Association. 2013. Diagnostic and statistical manual of mental disorders. 5th ed. Arlington, VA: American Journal of Psychiatry Publishing.
- Ashburner J, Friston KJ. 2000. Voxel-based morphometry—the methods. *Neuroimage*. 11:805–821.
- Baldelli P, Fassio A, Valtorta F, Benfenati F. 2007. Lack of synapsin I reduces the readily releasable pool of synaptic vesicles at central inhibitory synapses. *J Neurosci*. 27:13520–13531.
- Bogen IL, Jensen V, Hvalby O, Walaas SI. 2009. Synapsin-independent development of glutamatergic synaptic vesicles and presynaptic plasticity in postnatal mouse brain. *Neuroscience*. 158:231–241.
- Carper RA, Courchesne E. 2005. Localized enlargement of the frontal cortex in early autism. *Biol Psychiatry*. 57:126–133.
- Cesca F, Baldelli P, Valtorta F, Benfenati F. 2010. The synapsins: key actors of synapse function and plasticity. *Prog Neurobiol*.
- Chadman KK, Gong S, Scattoni ML, Boltuck SE, Gandhi SU, Heintz N, Crawley JN. 2008. Minimal aberrant behavioral phenotypes of neurologin-3 R451C knockin mice. *Autism Res*. 1:147–158.
- Chi P, Greengard P, Ryan TA. 2001. Synapsin dispersion and recluster during synaptic activity. *Nat Neurosci*. 4: 1187–1193.
- Chiappalone M, Casagrande S, Tedesco M, Valtorta F, Baldelli P, Martinoia S, Benfenati F. 2009. Opposite changes in glutamatergic and GABAergic transmission underlie the diffuse hyperexcitability of synapsin i-deficient cortical networks. *Cereb Cortex*. 19:1422–1439.
- Clarke DF, Roberts W, Daraksan M, Dupuis A, McCabe J, Wood H, Snead OC, Weiss SK. 2005. The prevalence of autistic spectrum disorder in children surveyed in a tertiary care epilepsy clinic. *Epilepsia*. 46:1970–1977.
- Cole MW, Anticevic A, Repovs G, Barch D. 2011. Variable global dysconnectivity and individual differences in schizophrenia. *Biol Psychiatry*. 70:43–50.
- Corradi A, Fadda M, Piton A, Patry L, Marte A, Rossi P, Cadieux-Dion M, Gauthier J, Lapointe L, Motttron L, et al. 2014. SYN2 is an autism predisposing gene: loss-of-function mutations alter synaptic vesicle cycling and axon outgrowth. *Hum Mol Genet*. 23:90–103.
- Courchesne E, Redcay E, Kennedy DP. 2004. The autistic brain: birth through adulthood. *Curr Opin Neurol*. 17:489–496.
- De Filippis B, Ricceri L, Laviola G. 2010. Early postnatal behavioral changes in the Mecp2-308 truncation mouse model of Rett syndrome. *Genes, Brain Behav*. 9:213–223. Di Martino A, Zuo XN, Kelly C, Grzadzinski R, Meneses M, Schvarcz A, Rodman J, Lord C, Castellanos FX, Milham MP. 2013. Shared and distinct intrinsic functional network centrality in autism and attention-deficit/hyperactivity disorder. *Biol Psychiatry*. 74:623–632.
- Dodero L, Damiano M, Galbusera A, Bifone A, Tsafaris SA, Scattoni ML, Gozzi A. 2013. Neuroimaging evidence of major morpho-anatomical and functional abnormalities in the BTBR T+TF/J mouse model of autism. *PLoS One*. 8(10):e76655.
- Dyck BA, Skoblenick KJ, Castellano JM, Ki K, Thomas N, Mishra RK. 2009. Behavioral abnormalities in synapsin II knockout mice implicate a causal factor in schizophrenia. *Synapse*. 63:662–672.
- Dyck BA, Tan ML, Daya RP, Basu D, Sookram CDR, Thomas N, Mishra RK. 2012. Behavioral effects of non-viral mediated RNA interference of synapsin II in the medial prefrontal cortex of the rat. *Schizophr Res*. 137:32–38.
- Ecker C, Murphy D. 2014. Neuroimaging in autism—from basic science to translational research. *Nat Rev Neurol*. 10:82–91.
- Esposito G, Venuti P, Apicella F, Muratori F. 2011. Analysis of unsupported gait in toddlers with autism. *Brain Dev*. 33:367–373.
- Farisello P, Boido D, Nieuws T, Medrihan L, Cesca F, Valtorta F, Baldelli P, Benfenati F. 2013. Synaptic and extrasynaptic origin of the excitation/inhibition imbalance in the hippocampus of synapsin I/II/III knockout mice. *Cereb Cortex*. 23:581–593.
- Fassio A, Patry L, Congia S, Onofri F, Piton A, Gauthier J, Pozzi D, Messa M, Defranchi E, Fadda M, et al. 2011. SYN1 loss-of-function mutations in autism and partial epilepsy cause impaired synaptic function. *Hum Mol Genet*. 20:2297–2307.
- Feng J, Chi P, Blanpied TA, Xu YM, Magarinos AM, Ferreira A, Takahashi RH, Kao HT, McEwen BS, Ryan TA, et al. 2002. Regulation of neurotransmitter release by synapsin III. *J Neurosci*. 22:4372–4380.
- Ferrari L, Turrini G, Crestan V, Bertani S, Cristofori P, Bifone A, Gozzi A. 2012. A robust experimental protocol for pharmacological fMRI in rats and mice. *J Neurosci Methods*. 204:9–18.
- Ferreira A, Kao HT, Feng J, Rapoport M, Greengard P. 2000. Synapsin III: developmental expression, subcellular localization, and role in axon formation. *J Neurosci*. 20:3736–3744.
- Fornasiero EF, Bonanomi D, Benfenati F, Valtorta F. 2010. The role of synapsins in neuronal development. *Cell Mol Life Sci*. 67(9):1383–1396.
- Gilby KL, O'Brien TJ. 2013. Epilepsy, autism, and neurodevelopment: kindling a shared vulnerability? *Epilepsy Behav*. 26(3): 370–374.
- Giovedì S, Corradi A, Fassio A, Benfenati F. 2014. Involvement of synaptic genes in the pathogenesis of autism spectrum disorders: the case of synapsins. *Front Pediatr*. 2:94.
- Gitler D, Xu Y, Kao H-T, Lin D, Lim S, Feng J, Greengard P, Augustine GJ. 2004. Molecular determinants of synapsin targeting to presynaptic terminals. *J Neurosci*. 24:3711–3720.
- Greco B, Manago F, Tucci V, Kao HT, Valtorta F, Benfenati F. 2013. Autism-related behavioral abnormalities in synapsin knockout mice. *Behav Brain Res*. 251:65–74.
- Haneef Z, Lenartowicz A, Yeh HJ, Levin HS, Engel J, Stern JM. 2014. Functional connectivity of hippocampal networks in temporal lobe epilepsy. *Epilepsia*. 55:137–145.
- He X, Doucet GE, Sperling M, Sharan A, Tracy JI. 2015. Reduced thalamocortical functional connectivity in temporal lobe epilepsy. *Epilepsia*. 56:1571–1579.
- Hirota T, Veenstra-Vanderweele J, Hollander E, Kishi T. 2014. Antiepileptic medications in autism spectrum disorder: a systematic review and meta-analysis. *J Autism Dev Disord*. 44:948–957.
- Holmes MJ, Yang X, Landman BA, Ding Z, Kang H, Abou-Khalil B, Sonmez Turk HH, Gore JC, Morgan VL. 2013. Functional networks in temporal-lobe epilepsy: a voxel-wise study of resting-state functional connectivity and gray-matter concentration. *Brain Connect*. 3:22–30.
- Janke AL, Ullmann J, Kumiawan N, Paxinos G, Keller M, Yang Z, Richards K, Egan G, Petrou S, Galloway GRD. 2012. 15 μm average mouse models in Waxholm space from 16.4T 30 μm images. In: 20th Annual ISMRM Scientific Meeting and Exhibition. Melbourne, Australia.
- Jeste SS, Tuchman R. 2015. Autism spectrum disorder and epilepsy: two sides of the same coin? *J Child Neurol*. 30: 1963–1971.
- Lakhan R, Kalita J, Misra UK, Kumari RMB. 2010. Association of intronic polymorphism rs3773364 A>G in synapsin-2 gene with idiopathic epilepsy. *Synapse*. 64:403–408.
- Levy SE, Giarelli E, Lee L-C, Schieve LA, Kirby RS, Cunniff C, Nicholas J, Reaven J, Rice CE. 2010. Autism spectrum disorder and co-occurring developmental, psychiatric, and medical conditions among children in multiple populations of the United States. *J Dev Behav Pediatr*. 31:267–275.
- Liska A, Galbusera A, Schwarz AJ, Gozzi A. 2015. Functional connectivity hubs of the mouse brain. *Neuroimage*. 115:281–291.
- Liska A, Gozzi A. 2016. Can mouse imaging studies bring order to autism connectivity chaos? *Front Neurosci*. 10:484.

- Liska A, Bertero A, Gomolka R, Sabbioni M, Galbusera A, Barsotti N, Panzeri S, Scattoni ML, Pasqualetti MGA. 2017. Homozygous loss of autism-risk gene CNTNAP2 results in reduced local and long-range prefrontal functional connectivity. *Cereb Cortex*. 10:1–13.
- Matsuo M, Maeda T, Sasaki K, Ishii K, Hamasaki Y. 2010. Frequent association of autism spectrum disorder in patients with childhood onset epilepsy. *Brain Dev*. 32:759–763.
- Medrihan L, Cesca F, Raimondi A, Lignani G, Baldelli P, Benfenati F. 2013. Synapsin II desynchronizes neurotransmitter release at inhibitory synapses by interacting with presynaptic calcium channels. *Nat Commun*. 4:1512.
- Medrihan L, Ferrea E, Greco B, Baldelli P, Benfenati F. 2015. Asynchronous GABA release is a key determinant of tonic inhibition and controls neuronal excitability: a study in the synapsin II-/- mouse. *Cereb Cortex*. 25:3356–3368.
- Michetti C, Romano E, Altabella L, Caruso A, Castelluccio P, Bede G, Gaetani S, Canese R, Laviola G, Scattoni ML. 2014. Mapping pathological phenotypes in reelin mutant mice. *Front Pediatr*. 2:95.
- Nguyen DK, Rouleau I, Senechal G, Ansaldo AI, Gravel M, Benfenati F, Cossette P. 2015. X-linked focal epilepsy with reflex bathing seizures: characterization of a distinct epileptic syndrome. *Epilepsia*. 56:1098–1108.
- Olmos-Serrano JL, Corbin JG, Burns MP. 2011. The GABA A receptor agonist THIP ameliorates specific behavioral deficits in the mouse model of fragile X syndrome. *Dev Neurosci*. 33(5):395–403.
- Pagani M, Bifone A, Gozzi A. 2016a. Structural covariance networks in the mouse brain. *Neuroimage*. 129:55–63.
- Pagani M, Damiano M, Galbusera A, Tsaftaris SA, Gozzi A. 2016b. Semi-automated registration-based anatomical labelling, voxel based morphometry and cortical thickness mapping of the mouse brain. *J Neurosci Methods*. 267:62–73.
- Phagava H, Muratori F, Einspieler C, Maestro S, Apicella F, Guzzetta A, Pechtl HFR, Cioni G. 2008. General movements in infants with autism spectrum disorders. *Georgian Med News*. 100–105.
- Picker JD, Yang R, Ricceri L, Berger-Sweeney J. 2006. An altered neonatal behavioral phenotype in Mecp2 mutant mice. *Neuroreport*. 17:541–544.
- Pittau F, Grova C, Moeller F, Dubeau F, Gotman J. 2012. Patterns of altered functional connectivity in mesial temporal lobe epilepsy. *Epilepsia*. 53:1013–1023.
- Porton B, Wetsel WC, Kao H-T. 2011. Synapsin III: role in neuronal plasticity and disease. *Semin Cell Dev Biol*. 22:416–424.
- Romano E, Michetti C, Caruso A, Laviola G, Scattoni ML. 2013. Characterization of neonatal vocal and motor repertoire of reelin mutant mice. *PLoS One*. 8(5):e64407.
- Santos M, Uppal N, Butti C, Wicinski B, Schmeidler J, Giannakopoulos P, Heinsen H, Schmitz C, Hof PR. 2011. von Economo neurons in autism: a stereologic study of the fronto-insular cortex in children. *Brain Res*. 1380:206–217.
- Scattoni ML, Gandhi SU, Ricceri L, Crawley JN. 2008. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS One*. 3(8):e3067.
- Scattoni ML, Ricceri L, Crawley JN. 2011. Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social encounters. *Genes, Brain Behav*. 10:44–56.
- Schumann CM, Hamstra J, Goodlin-Jones BL, Lotspeich LJ, Kwon H, Buonocore MH, Lammers CR, Reiss AL, Amaral DG. 2004. The amygdala is enlarged in children but not adolescents with autism; the hippocampus is enlarged at all ages. *J Neurosci*. 24:6392–6401.
- Sforzini F, Bertero A, Dodero L, David G, Galbusera A, Scattoni ML, Pasqualetti M, Gozzi A. 2016. Altered functional connectivity networks in acallosal and socially impaired BTBR mice. *Brain Struct Funct*. 221:941–954.
- Sforzini F, Schwarz AJ, Galbusera A, Bifone A, Gozzi A. 2014. Distributed BOLD and CBV-weighted resting-state networks in the mouse brain. *Neuroimage*. 87:403–415.
- Teitelbaum O, Benton T, Shah PK, Prince A, Kelly JL, Teitelbaum P. 2004. Eshkol-Wachman movement notation in diagnosis: the early detection of Asperger's syndrome. *Proc Natl Acad Sci U S A*. 101:11909–11914.
- Uddin LQ, Supekar K, Menon V. 2013. Reconceptualizing functional brain connectivity in autism from a developmental perspective. *Front Hum Neurosci*. 7:458.
- van Steensel FJA, Bögels SM, Perrin S. 2011. Anxiety disorders in children and adolescents with autistic spectrum disorders: a meta-analysis. *Clin Child Fam Psychol Rev*. 14(3):302–317.
- Vasa RA, Mostofsky SH, Ewen JB. 2016. The disrupted connectivity hypothesis of autism spectrum disorders: time for the next phase in research. *Biol Psychiatry Cogn Neurosci Neuroimaging*. 1(3):245–252.
- Waites AB, Briellmann RS, Saling MM, Abbott DF, Jackson GD. 2006. Functional connectivity networks are disrupted in left temporal lobe epilepsy. *Ann Neurol*. 59:335–343.
- Woolfenden S, Sarkozy V, Ridley G, Coory M, Williams K. 2012. A systematic review of two outcomes in autism spectrum disorder—epilepsy and mortality. *Dev Med Child Neurol*. 54:306–312.