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Subconvulsant doses of pentylenetetrazol uncover the epileptic phenotype of cultured synapsin-deficient *Helix* serotonergic neurons in the absence of excitatory and inhibitory inputs

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Abstract

Synapsins are a family of presynaptic proteins related to several processes of synaptic functioning. A variety of reports have linked mutations in synapsin genes with the development of epilepsy. Among the proposed mechanisms, a main one is based on the synapsin-mediated imbalance towards network hyperexcitability due to differential effects on neurotransmitter release in GABAergic and glutamatergic synapses. Along this line, a non-synaptic effect of synapsin depletion increasing neuronal excitability has recently been described in *Helix* neurons. To further investigate this issue, we examined the effect of synapsin knock-down on the development of pentylenetetrazol (PTZ)-induced epileptic-like activity using single neurons or isolated monosynaptic circuits reconstructed on microelectrode arrays (MEAs). Compared to control neurons, synapsin-silenced neurons showed a lower threshold for the development of epileptic-like activity and prolonged periods of activity, together with the occurrence of spontaneous firing after recurrent PTZ-induced epileptic-like activity. These findings highlight the crucial role of synapsin on neuronal excitability regulation in the absence of inhibitory or excitatory inputs.

Keywords

Synapsin; pentylenetetrazol (PTZ); invertebrate neurons; convulsants; *Helix* snail.

Highlights

- Synapsin-silenced cells lacking synaptic inputs exhibit greater PTZ susceptibility.
- PTZ-induced epileptic-like behaviors were increased in synapsin-silenced cells.
- Recurrent epileptic-like activity impairs control neuron synaptogenesis.
- Recurrent epileptic-like activity is associated with spontaneous spiking activity.

Abbreviations

AP, action potential

ISI, interspike interval

PDS, paroxysmal depolarization shift

Syn, synapsin

TKO, triple knock-out

PTZ, pentylenetetrazol

MEA, microelectrode array

helSynKD, *Helix* synapsin knock-down

CC, cross-correlogram

Introduction

Synapsins (Syn) are a family of evolutionarily conserved presynaptic proteins, crucial for the fine-tuning of synaptic transmission, neuritic outgrowth, and synapse formation and remodeling (Cesca et al., 2010). Experimental evidence shows that Syns are involved in the development of epilepsy (Li et al., 1995; Rosahl et al., 1995; Gitler et al., 2004; Etholm and Heggelund, 2009; Ketzef et al., 2011; Etholm et al., 2013; Ketzef and Gitler, 2014). In mice, most single, double, and triple knock-outs (TKO) for Syn genes lead to a severe epileptic phenotype, with a progressive increase in the frequency of seizures with time (Li et al., 1995; Gitler et al., 2004; Cesca et al., 2010; Fassio et al., 2011a).

Epileptic activity is visualized as abnormal neuronal bursting and epileptic discharges that appear in the form of (1) instability and oscillations of the resting membrane potential, (2) high frequency spike discharges, including action potential (AP) doublets with short interspike intervals (ISI), and (3) paroxysmal depolarization shifts (PDS), consisting of steep

depolarizations followed by a plateau potential with superimposed APs that terminate with a steep repolarizations (Klee, 1976; Altrup, 2004).

To analyze abnormal epileptic discharges, epileptic activity is usually induced through convulsant drugs, such as pentylenetetrazol (PTZ), which is widely employed in both vertebrate and invertebrate models to investigate the degree of hyperexcitability of neuronal networks and seizures. The neurons of *Helix* land snails have been used frequently to studying the mechanisms underlying drug-induced epileptic-like activity (Speckmann and Caspers, 1973; Altrup and Speckmann, 1988; Madeja et al., 1989; Altrup et al., 1991; Altrup et al., 2003; Altrup et al., 2006; Üre and Altrup, 2006), mimicking those described in mammalian neurons (Chalazonitis and Takeuchi, 1968; Speckmann and Caspers, 1973). More recently the isolated *Helix* neurons have also been used to study the effects of the drug-induced epileptiform activity on basal synaptic transmission and post-tetanic potentiation (Giachello et al., 2013).

Cultured *Helix* neurons (Ghirardi et al., 1996) offer great advantages for studying neuronal activity and epileptic-like patterns. They are identifiable, form monosynaptic connections *in vitro* (Fiumara et al., 2001; Fiumara et al., 2005), and allow a variety of experimental manipulations that are not feasible with mammalian neurons, such as single cell-electrode coupling on microelectrode arrays (MEAs) (Massobrio et al., 2009; Massobrio et al., 2013) and intranuclear DNA injection (Brenes et al., 2015a; Brenes et al., 2015b). The convenient genetic organization (one single *Syn* gene) of this organism allows antisense RNA (asRNA)-dependent constitutive Syn depletion without cellular compensatory mechanisms. In addition, the possibility of culturing single or paired cells with well-identified monosynaptic connections avoids non-specific effects due to signals from undesired surrounding tissues.

Recently, we used *Helix* neurons as a model system for studying the morphological and functional effects of Syn knock-down (Brenes et al., 2015a; Brenes et al., 2015b). In Syn-silenced neurons we saw impairment of synaptogenesis, fast high-frequency neurotransmitter release, and increased excitability with changes in Ca^{2+} and K^{+} currents in Syn-silenced

neurons. In this work, by using intracellular and MEA recordings, we tested how Syn knock-down affects neuronal susceptibility to the PTZ-induced epileptic-like discharges. Using this convulsant drug we uncovered their lower epileptic threshold and highlighted the hyperexcitable phenotype of Syn-silenced neurons.

Materials and Methods

Materials

All chemicals and reagents used in this study were purchased from Sigma (Milan, Italy) unless stated otherwise.

Animals

Juvenile specimens of *Helix aspersa* land snails were provided by local breeders and kept as previously described (Brenes et al., 2015a). Efforts were made to minimize the number and suffering of animals used, and experiments were conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Cell culture

Cell cultures were performed as previously described by Ghirardi et al. (1996) and modified according to Brenes et al. (2015a). Briefly, snails were anesthetized with $MgCl_2$, ganglia were surgically isolated and incubated for enzymatic digestion in protease type XIV (0.3 U/mL) at 34°C for 3-3.5 h. Neurons were individually isolated and transferred to dishes pretreated with 5% bovine serum albumin to prevent cell-substrate adhesion. After 24 h, floating neurons retracted their processes, obtaining spherical somata (soma-configuration) (Fiumara et al., 2005). Floating somata were then gently manipulated according to the specific experimental protocols.

Plasmid generation and intranuclear microinjection

As previously described (Brenes et al., 2015a; Brenes et al., 2015b), the $pNEX_3$ plasmid containing the EGFP sequence (pNEX-EGFP) was used as a control and standard recombinant DNA techniques were used to construct an asRNA-expressing plasmid against *Helix* Syn. Control neurons were intranuclearly microinjected with pNEX-EGFP alone, whereas knocked-down neurons (helSynKD) were injected with both plasmids. Intranuclear microinjection and immunocytochemical analyses to estimate Syn protein presence were performed as previously described (Brenes et al., 2015a). Experiments were performed on neurons expressing the asRNA for 48 h and 72 h that presented a marked decrease in Syn immunostaining, as previously reported (Brenes et al., 2015a; Brenes et al., 2015b).

Intracellular electrophysiological recordings

Standard intracellular recording techniques were used on single cultured cells as previously described (Fiumara et al., 2005; Fiumara et al., 2007). Briefly, the cells were impaled with intracellular electrodes, filled with 2.5 M KCl (resistance ~10 MΩ). Signals were amplified by an Axoclamp 900A amplifier (Axon Instruments, Union City, CA, USA) in the current clamp mode and monitored and recorded using Axoscope software (Axon Instruments) on a personal computer. The recorded traces were analyzed with Clampfit software (Axon Instruments).

Microelectrode Array (MEA) experimental setup

Helix C1 and B2 neurons were plated individually over MEA devices previously coated with poly-L-lysine and *Aplysia* hemolymph. The experimental data were collected from seven different experiments consisting of two or three MEA chips in each one. *Helix* cultures on MEAs were followed-up for 72 h and recordings were performed using an MEA-system (Multi-Channel Systems, Reutlingen, Germany) with a 60-electrode array (TiN/SiN), composed of a grid with 200 µm inter-electrode spacing and 30 µm electrode diameter (Gavello et al., 2012). Data acquisition was controlled through MC_Rack software (Multi-Channel Systems, Reutlingen, Germany), sampling at 10 kHz (Allio et al., 2015). The recorded traces were analyzed with Clampfit software (Axon Instruments) (Gavello et al., 2012; Allio et al., 2015).

PTZ treatments and recordings

PTZ was dissolved in L-15 medium at two subconvulsive concentrations (10 and 20 mM) and at the reported epileptogenic dose (40 mM) (Altrup and Speckmann, 1988; Altrup, 2004; Altrup et al., 2006; Giachello et al., 2013). Isolated C1 cells plated on culture dishes or C1-B2 pairs plated on MEAs were perfused with the PTZ solutions using a peristaltic pump (Ismatec ISM829, Glattbrugg, Switzerland). Single cell intracellular recordings were performed for 15 min in the presence of the drug. With extracellular MEA recordings the stimulation protocol consisted of 1) neurons were allowed to stabilize for 10 min, then spontaneous activity was recorded for 10 min; 2) PTZ was perfused and the activity was recorded for 15 min; 3) the drug was washed out with 10 mL of fresh medium (8.3 times the MEA volume) and the remaining activity was recorded for 10 min.

PTZ treatments were performed at 24 h, 48 h, and 72 h after plating in the same neuronal cultures, following cell populations throughout sequential recordings in time.

Measurement of neurite density

The quantification of the density of neurites in the space between electrodes was performed as previously described (Massobrio et al., 2013). Briefly, bright field images of MEA cultures were acquired at 24 h, 48 h, and 72 h after plating. The neurite density between pairs of C1 and B2 neurons was assessed by counting the total number of neurites crossing a box of fixed area (40000 μm^2) drawn equidistant between the two neurons and dividing by the surface area. Values were normalized by the dendrite density of the control untreated neurons at 24 h after plating.

Statistical analysis

Data are expressed as the mean ± standard error of mean. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). Kolmogorov-Smirnov and Shapiro-Wilk tests were used as normality tests and the F-test was used to compare variances. Significant differences between two or more groups were assessed using non-

parametric (Kruskal-Wallis tests followed by the Dunn's multiple comparison test) or parametric tests (Pearson's chi-square test, Student's t-test, or two-way ANOVA followed by the Bonferroni post-hoc test) when appropriate. For repeated measures data the square root transformation was used to normalize data and a mixed ANOVA with a multilevel model was used. The level of significance was set at $P < 0.05$.

Results

Responsiveness of helSynKD neurons to PTZ

In control conditions, C1 *Helix* neurons do not exhibit firing activity at rest. Bath applications of the epileptogenic drug PTZ at two subconvulsive concentrations (10 and 20 mM) and at an epileptogenic dose (40 mM) were therefore used to test the predisposition of resting helSynKD and control neurons to elicit discharges associated with epileptic phenotypes, such as spiking, AP doublets, and PDSs (Fig. 1A). The susceptibility of neurons was estimated by evaluating the percentage of cells that responded with abnormal epileptic-like discharges to the drug application, the latency and the duration of the response as a percentage of the total recording period.

A high percentage of helSynKD neurons responded to PTZ in a concentration-dependent manner (Fig. 1B), relative to control neurons. At the lowest dose (10 mM), the difference was maximal between helSynKD and control neurons and statistically significant. Indeed, while a limited number of control neurons responded to 10 mM PTZ (30.8%, n = 13), the majority of helSynKD (81.8%; n = 11) responded to the same concentration ($\chi^2_{(1)}$: 6.25, $P < 0.05$). Application of 20 mM PTZ enhanced the percentage of control neurons that developed epileptiform activity (50.0%, n = 16), while the percentage remained unchanged in helSynKD neurons (80.0%, n = 15) ($\chi^2_{(1)}$: 3.04, $P = 0.08$). The epileptic dose (40 mM) affected both groups, evoking abnormal activity in most of the control as well as helSynKD neurons (71.4%, n = 14; and 90.9%, n = 11, respectively) ($\chi^2_{(1)}$: 1,46, $P = 0.23$).

In agreement with an increased susceptibility of Syn-silenced neurons to develop epileptic-like behavior, the latency to onset of the drug-induced discharges was shorter in helSynKD neurons than that in controls and in both groups decreased with increasing PTZ concentrations (Fig. 1C). In helSynKD neurons the latency was 59.4% shorter with 10 mM PTZ treatment, 65.4% shorter with 20mM, and 81.8% shorter with 40 mM ($K = 29.82$, $P < 0.0001$).

Similarly, compared to control neurons, helSynKD neurons showed longer periods of activity (Fig. 1D) and the difference was maximal at the lowest PTZ dose ($K = 23.57$, $P = 0.0003$). With 10 mM PTZ, helSynKD neurons showed a 10-fold increase in average activity duration compared to control neurons. With 20 mM and 40 mM PTZ there was tendency to longer periods of activity, with a 2.5 and 2.6-fold increase in the duration of helSynKD neuron activity compared to control neurons.

Epileptic-like activity characterization in single Syn-silenced cells

Previous experiments suggested that the mechanisms of PTZ-induced epileptiform activity include inactivation of K^+ currents and the development of a sustained inward Na^+/Ca^{2+} currents (Onozuka et al., 1983; Walden et al., 1988; Fehér et al., 1988; Onozuka et al., 1991; Onozuka and Tsujitami, 1991). The PTZ-induced activity is characterized by a steady depolarization and three main patterns of AP firing: fast tonic activity, AP doublets and, PDSs (Klee, 1976; Altrup, 2004), which were observed in both control and helSynKD neurons (Fig. 1A). The analysis of the percentage of treated cells that developed these patterns of activity underscored the capacity of Syn-silenced neurons to develop epileptic-like activity. This effect was more evident at low PTZ concentrations, at which 3-fold more helSynKD neurons (n = 10) than control neurons (n = 12) developed PDSs ($\chi^2_{(1)}$: 4.11, $P = 0.04$). In addition, no control neurons developed doublets and twice as many Syn-silenced neurons developed AP spiking ($\chi^2_{(1)}$: 6.25, $P = 0.01$) (Fig. 2A) when treated with 10 mM PTZ. At increasing drug concentrations (20 and 40 mM; Fig. 2B and 2C) a larger number of control neurons (n = 16 and 14, respectively) developed these activity patterns. Thus, the percentage of cells exhibiting epileptic-like activity

was already at a maximal level in helSynKD neurons treated with the lowest drug concentration (10 mM), while the epileptic-like activity in control neurons was at a minimum and increased progressively in a drug concentration-dependent fashion.

Besides evaluating the percentage of cells exhibiting epileptic-like activity, we monitored the duration of each type of activity at increasing PTZ doses (Fig 2D-F). The analysis showed that the dominant activity in the Syn-silenced neurons was the fast tonic activity at all tested PTZ concentrations. When treated with 10 mM PTZ helSynKD neurons developed the three epileptic-like behaviors and the biggest difference regarding control neurons was observed for spiking behavior ($K = 21.66$, $P = 0.0006$). In particular, AP spiking represented 36.3% and paroxysmal activity 20.4% of the total recording periods, compared to 4.1% and 0.7% in control neurons (Fig 2D and F). At higher PTZ concentrations (20 and 40 mM) the activity duration of doublets and PDS was similar between control and helSynKD neurons, but at these concentrations there was a 3-fold and an statistically significant 10-fold increase in fast tonic activity in helSynKD neurons over that in control neurons (Fig. 2D). There was no statistical differences in spiking or PDS frequency between groups or drug concentrations. Only at the highest PTZ concentration doublet frequency was 3-fold greater in helSynKD than in control neurons (data not shown).

Epileptic-like activity induced by PTZ in Syn-silenced C1 paired with B2 neurons

MEAs provide a powerful tool for the functional reconstruction of invertebrate neuron synapses. Indeed, specific neurons, connected *in vivo* can be isolated and plated on adjacent electrodes and their excitability monitored for days. In order to assess how recurrent epileptic-like activity influences cell excitability, neurite outgrowth and functional synapse formation in the Syn-silenced model, C1 neurons and their physiological targets (B2) were plated on MEAs according to the configuration illustrated in Figure 3A. Since B2-B2 synapses developed *in vitro* (Giachello et al., 2010), in each array we coupled the neurons C1-B2 on two contiguous microelectrodes

far away from other pairs of neurons. In this way we kept each monosynaptic circuit independent of neighboring cells.

Few hours after plating, the paired cells developed neurites that established local contacts between them (Fig. 3B). Epileptic-like activity (Fig. 3C) was sequentially induced by 40 mM PTZ in the same cell cohorts at 24 h, 48 h and 72 h and the neuronal activity (spontaneous and drug-induced) was monitored. Data were obtained from seven different experiments with two or three MEAs each.

Most drug-induced activity had the typical shape of extracellular recordings, i.e., fast inward deflection followed by a slow outward deflection that corresponds to the negative of the first derivative of intracellular AP (Fig. 3C) (Gavello et al., 2012; Vandael et al., 2010; Henze et al., 2000). Sometimes the extracellular waveform consisted of a large positive deflection which is likely due to the differential proximity and coupling of the electrode to the neuronal cell body (Henze et al., 2000).

As observed in single-cell intracellular recordings, compared to control neurons, the Syn-silenced neurons exhibited a greater tendency to develop epileptic-like activity under PTZ treatment (Fig. 4A-B). About 50% of C1 control neurons responded to PTZ treatment regardless of the time after cell plating (n = 22 at 24 h, n = 20 at 48 h, and n = 17 at 72 h). In contrast, the percentage of helSynKD neurons that developed abnormal activity was higher than control neurons (n = 17 at 24 h, n = 15 at 48 h, and n = 12 at 72 h) and increased over time from 64.7% at 24 h to 83.3% at 72 h (Fig. 4B). The increased excitability of Syn-silenced neurons over that of controls was also evident after drug washout. On average 29.1% of helSynKD neurons maintained their firing after PTZ washout versus a 3.7% of control neurons (data not shown). In addition, *Helix* neurons rarely showed spontaneous firing when cultured on MEAs, as previously reported (Massobrio et al., 2009; Massobrio et al., 2013). In our experiments, 24 h after cell plating on MEAs, and before the first PTZ treatment, no spontaneous activity was recorded. However, at 48 h after plating, before the second PTZ treatment, spontaneous neural activity

was evident in both helSynKD and control neurons. At 72 h the spontaneous electrical activity was 3-fold more evident in Syn-silenced neurons (33.3%) than in control neurons (11.8%) (Fig. 4C).

Analyzing the different firing patterns on MEAs, neurons presenting doublets were rare or this activity was alternated with single spike firing, and no clear patterns were observed in the percentage of cells that developed spikes or PDS up to 72 h (Fig. 5A-B). The duration of spiking activity showed a tendency to increase with time in helSynKD neurons with the stronger effect at 72 h after plating (Fig. 5C), an effect not evident in control neurons. However, there were not significant main effects of the Syn-silencing ($\chi^2_{(1)}$: 2.40, P = 0.12). The PDS activity duration was stable, independent of the repetitive drug exposure through 72 h (Fig. 5D), and slightly bigger in helSynKD neurons, however the main effects of the Syn-silencing were not significant ($\chi^2_{(1)}$: 4.90, P = 0.08). The frequencies were also similar between control and helSynKD neurons regardless of the time after cell plating, both in terms of spiking (1.1 ± 0.4 Hz in control vs. 1.6 ± 1.2 Hz in helSynKD) and PDS (0.3 ± 0.3 Hz in control vs. 1.0 ± 0.5 Hz in helSynKD). Even though spike frequency within each PDS was slightly higher in helSynKD neurons, the difference between groups was not statistically significant (4.7 ± 0.5 Hz in control vs. 5.9 ± 0.8 Hz in helSynKD).

Previous studies described a potentiation of electrically-evoked responses in mouse Syn TKO slices (Ketzef et al., 2011). To further analyze the excitability of cultured Syn-silenced *Helix* neurons, we measured the electrically-evoked responses following biphasic electrical stimulation before PTZ treatment (pretr) and during PTZ treatment in neurons in which no spontaneous or drug-induced activities were observed at 24 h and 48 h. Electrically- and PTZ-induced activities were similar. In the first 24 h after plating (Fig. 5E), the activity evoked in control neurons by a single stimulation was very short in duration with or without PTZ in the medium. In helSynKD neurons spiking activity was observed for 50-90% of the total recording time (silencing $F_{(1,10)} = 122.1$, $P < 0.0001$; PTZ treat $F_{(1,10)} = 17.96$, $P = 0.02$; and interaction

$F_{(1,10)}$ = 8.74, P = 0.01; two-way ANOVA). At 48 h (Fig. 5F), control neurons showed increased excitability since the total duration of the activity after the electrical stimulation increased in the absence and the presence of PTZ. On the other hand, Syn-silenced neurons kept their high excitability state from 24 h to 48 h, with a modest increase in both conditions.

All together, these results emphasize the existence of increased excitability in Syn-silenced neurons. Silencing of Syn makes the neurons more prone to drug-induced epileptic activity than are control neurons. The increased excitability of Syn-silenced *Helix* neurons could be ascribed to different mechanisms of action on Ca^{2+} and K^+ currents, with increased Ca^{2+} and Ca^{2+}-dependent K^+ currents in Syn-deficient neurons (Brenes et al., 2015b). In addition, the suppression of K^+ currents and activation of inward currents partially carried Ca^{2+}, have been observed following PTZ application in different models (Onozuka et al., 1983; Walden et al., 1988; Fehér et al., 1988; Onozuka et al., 1991; Onozuka and Tsujitami, 1991). From all these reports, we can reasonably conclude that if PTZ induces K^+ currents reduction and Ca^{2+} currents increase in helSynKD cells, which present increased Ca^{2+} currents, it could lead to an augmented depolarization and increased excitability in helSynKD neurons. This possibility, however, requires future clarification.

PTZ modulation of synaptogenesis in Syn-silenced C1 paired with B2 neurons on MEAs

It has been suggested that epileptic activity can affect the structural morphology of neurons and their electrical properties, leading to axonal sprouting, dendritic destruction and increased excitability (Meilleur et al., 2003; Altrup, 2004). Thus, we applied recurrent PTZ treatments (at 24 h, 48 h, and 72 h after cell plating) at an epileptogenic doses (40 mM) and analyzed the presence, type, and strength of the synaptic contacts developed by the paired cells by cross-correlating the activity in pre- and postsynaptic neurons.

When *Helix* neurons are coupled 1:1 to microelectrodes, the MEA system allows an easy assessment of synaptic functionality and cross-correlograms (CC) can be used to characterize and classify the functional synapses developed between paired *Helix* neurons, as previously

reported by Massobrio et al. (2009; 2013). Functional synapses were classified as chemical or electrical depending on the delay between the pre- and post-synaptic cell responses.

The activity recorded on MEAs, both spontaneous and drug-induced, was mainly *asynchronous* (Fig. 6A). The delay between pre- and postsynaptic spikes was similar in control and helSynKD paired cells, around 26.5 ms ($P = 0.85$, t-test) (Fig 6B and C), suggesting the presence of chemical connections in both conditions (Massobrio et al., 2013). The strength of the chemical connections was similar between control and helSynKD neurons, with a slight increase in Syn-silenced neurons compared to control neurons, although the difference was not statistically significant ($P = 0.22$, t-test) (Fig. 6B and D). This weak coupling between paired neurons could explain the low-level of synchronicity of cell firing observed in these synaptic connections.

On the MEAs, functional synapses were observed in 35.7% of the helSynKD neurons (Fig. 6E). A similar percentage was previously reported in Syn-silenced PTZ untreated C1-B2 cell pairs (Brenes et al., 2015a). Control neurons developed a comparable number of functional connections (40.9%), although previous reports showed that untreated control neurons formed synapses in 81.5% of the C1-B2 pairs (Brenes et al., 2015a). The number of synaptic connections measured as a function of network development after cell plating was also similar between control and Syn-silenced neurons. Taken together these results suggest that although synaptogenesis in helSynKD neurons is not further impaired under recurrent drug-induced epileptic-like activity, in control neurons the recurrent epileptic activity strongly affects synaptogenesis.

Alteration of neuronal structure after drug-induced epileptogenic conditions has been reported (reviewed in Altrup, 2004). Impaired synaptogenesis in control neurons could be related to changes in neurite density. Therefore, the neuritic density between C1-B2 paired cells was quantified (Fig. 7A). Paired control neurons presented similar neuritic density through time, independently of PTZ treatments (Fig. 7B). PTZ-treated Syn-silenced neurons showed a lower neurite density than controls (treatment $F_{(2,204)} = 4.05$, $P < 0.05$; time $F_{(3,204)} = 34.31$, $P < 0.0001$;

and interaction $F_{(6,204)} = 0.70$, $P = 0.65$; two-way ANOVA), as previously reported (Brenes et al., 2015a). However, similar to control neurons, there was no differences by comparing the neurite density of treated and non-treated helSynKD neurons 72 h after plating (Fig. 7B, insert). These findings suggest that lower neurite density in helSynKD neurons is most likely related to the Syn downregulation instead of PTZ-induced effects.

Discussion

In the present study, we analyzed the susceptibility of Syn-silenced neurons to develop drug-induced epileptic-like activity using intracellular recordings in single cells and extracellular recordings in monosynaptic circuits of *Helix* neurons using MEAs. The importance of characterizing the excitable phenotype developed by Syn deficiencies is highlighted by the fact that Syn deletion and mutations have been associated with the development of epileptic phenotypes in animals (Li et al., 1995; Rosahl et al., 1995; Gitler et al., 2004; Etholm and Heggelund, 2009; Ketzef et al., 2011; Etholm et al., 2013; Ketzef and Gitler, 2014). In humans, epilepsy and other disorders have been linked to mutations in *SynI* (Garcia et al., 2004; Fassio et al., 2011b; Lignani et al., 2013; Giannandrea et al., 2013) and *SynII* genes (Cavalleri et al., 2007; Lakhan et al., 2010; Corradi et al., 2014). In addition, the seizure susceptibility of mice TKO brain slices has been reported to anticipate epileptic phenotypes (Boido et al., 2010; Feliciano et al., 2013).

The nervous system of *Helix* has been proposed as a suitable model to study epileptogenicity, and to test epileptic or antiepileptic effects of different compounds (Altrup, 2004). Furthermore, it has been extensively studied concerning its electrophysiological (Chalazonitis and Takeuchi, 1968; Schulze et al., 1975; Altrup et al., 1991), morphological (Steffens, 1980), functional (Fiumara et al., 2004; Ghirardi et al., 2004; Fiumara et al., 2005; Fiumara et al., 2007; Massobrio et al., 2009; Giachello et al., 2010; Massobrio et al., 2013) and epileptological properties (Speckmann and Caspers, 1973; Altrup and Speckmann, 1988; Madeja et al., 1989;

Altrup et al., 1991; Schulze-Bonhage et al., 1993; Altrup et al., 2003; Üre and Altrup, 2006; Giachello et al., 2013).

In the present work, Syn silencing was achieved through intranuclear injection of a plasmid that allows the expression of an asRNA against *Helix* Syn. Possible non-specific effects were ruled out by analyzing sequence complementarity and potential cellular targets through BLAST Alignment; where no potential targets were found outside of Syn mRNA. The utilization of scrambled asRNA was excluded to prevent possible off-target effects of the scrambled sequence in *Helix* neurons. The possibility that the increased excitability was an effect of the asRNA blocking the expression of ion channel is very unlikely since the increased excitability is related with increased Ca^{2+} and Ca^{2+}-dependent K^+ currents (Brenes et al., 2015b).

In cultured *Helix* neurons, the epileptiform activity was evoked by PTZ application. PTZ induces epileptic activity in cultured *Helix* monosynaptic circuits, as reported by Giachello et al. (2013), and in single neurons, as we showed in the present study. Several mechanisms of action have been reported for PTZ in different cell models. In invertebrate neurons it has been suggested that PTZ inactivates K^+ currents, by a mechanism involving Ca^{2+}/calmodulin and PKA phosphorylations. According to this action, PTZ reduces the voltage- and Ca^{2+}-dependent K^+ currents (Kv and BK) and evokes an non-specific inward current (Na^+ and Ca^{2+}), which is responsible for maintaining sustained depolarizations (Onozuka et al., 1983; Walden et al., 1988; Fehér et al., 1988; Onozuka et al., 1991; Onozuka and Tsujitami, 1991). Also mammalian K^+ currents through Kv are depressed by PTZ at slightly negative and positive potentials (Madeja et al., 1996) and mice lacking Ca^{2+} channels, such as Cav2.3 (R-Type), are less susceptible to PTZ-induced seizure activity (Weiergräber et al., 2006). Additionally, in mammalian chromaffin cells, it has been reported that reduced Kv and BK channel activation results in a slower return to baseline, and this effect, together with an inward current carried by Ca^{2+}, triggers burst firing (Vandael et al., 2015).

Thus, in helSynKD neurons, in which Cav currents are increased (Brenes et al., 2015b), it is possible that the marked blocking of Kv and BK channels by PTZ application could leads to increased susceptibility to developing epileptic-like behavior, in the form of spiking, AP doublets and PDSs. The increased excitability causes also the shorter latencies and the longer periods of activity observed in Syn-silenced neurons compared to control neurons. However, the specific mechanism requires future clarification.

Moreover, the predisposition of helSynKD neurons to exhibit epileptic-like activity in the absence of excitatory and inhibitory inputs is supported by the higher percentage of Syn-silenced cells that respond at 10 mM PTZ (25% of the reported convulsive dose) (Altrup, 2004; Giachello et al., 2013). Following this treatment, a greater proportion of helSynKD neurons developed all the phenotypes of epileptic activity (i.e., AP spiking, doublets and PDS) than control neurons, exhibiting a lower threshold for the epileptic behavior in single cells.

Interestingly, brain slices of TKO adult mice have been reported to be more resistant to the epileptogenic drug 4-AP than WT adult mice. This apparently conflicting result could be related to the possible development of compensatory mechanisms in Syn TKO mice, such as strengthening of inhibitory GABAergic tone (Ketzef et al., 2011). In our case, monosynaptic connections were developed in the absence of inhibitory inputs, so their hyper-excitable state was uncovered by administration of PTZ.

MEAs have proven to be a useful device for studying network dynamics in mammalian and invertebrate circuits (Gavello et al., 2012; Vandael et al., 2010; Massobrio et al., 2015). This technique allowed us to record the activity from well-identified neurons over the course of 72 h in a noninvasive way. As a result, repeated and prolonged recordings induced no damage to cellular membranes, thus non-specific effects on ionic currents and membrane permeability were prevented. Here, MEAs were used to measure monosynaptic connection dynamics of control and helSynKD neurons under recurrent drug-induced epileptic-like activity.

Murine SynI-KO neurons cultured on MEAs presented activity rates higher than control neurons at early stages of development that further increased with network development. Their spontaneous activity was highly synchronized with respect to control neurons (Chiappalone et al., 2009). In our case, under control conditions, *Helix* neurons did not fire APs at rest, but they exhibited strong responses when exposed to PTZ. In addition, recurrent treatment with PTZ induced the appearance of spontaneous AP firing, which was more prominent in Syn-silenced neurons. Since spontaneous firing activity is dependent on the type and density of the ion channels expressed in the cell (Kandel et al., 2013), our results suggest that the activity induced by recurrent PTZ application could have been affecting the handling of channels in the membrane and further changing neuronal excitability in both control and Syn-silenced neurons. Connectivity was not significantly different between control and helSynKD neurons repeatedly treated with PTZ. In previous reports we showed that control *Helix* neurons have higher synaptogenic capacities than Syn-silenced neurons, developing functional chemical synapses in 81.5% of the paired neurons compared to 40.7% in helSynKD neurons (Brenes et al., 2015a). Impaired synaptogenesis could be related with the development of a lower number of varicosities and smaller in size in synapsin-silenced neurons (Brenes et al., 2015a). In the present work, we observed similar neuronal connectivity in control and helSynKD neurons that could be an effect of impaired synaptogenesis in control neurons induced by recurrent epileptic activity. In previous experiments, we also showed that Syn silencing impaired neurite linear outgrowth and branching (Brenes et al., 2015a). It is possible to infer that smaller neuritic fields could affect the number of functional synapses developed. Therefore, we compared neurite density during the recurrent drug treatments. The development of the neurite arbor between C1 and B2 was similar in treated and untreated control neurons. The neurite density after 72 h of plating was similar for control and helSynKD neurons independent of drug applications. These findings suggest that recurrent epileptic-like activity does not affect neurite arbor in our model, and the lower number of synapses in control neurons could reflect a functional effect. However,

the mechanisms behind these differences need to be investigated in more details in future studies.

It is generally accepted that Syn deficiency reduces GABAergic transmission and not affects or increases glutamatergic transmission, thus leading to a positive imbalance towards hyperexcitability (Terada et al., 1999; Gitler et al., 2004; Baldelli et al., 2007; Chiappalone et al., 2009; Ketzef et al., 2011; Farisello et al., 2012; Lignani et al., 2013; Feliciano et al., 2013; Medrihan et al., 2013; Medrihan et al., 2014). Our present results extend this largely accepted model, showing that Syn silencing increases cellular excitability in single neurons and monosynaptic circuits in the absence of excitatory and inhibitory inputs. Syn deficiency increases cellular susceptibility to develop drug-induced epileptic-like activity.

In addition, with this work, we presented the excitability properties of a monosynaptic cell system made of primary cultures of *Helix* neurons that can be useful for experiments on epileptogenic or anti-epileptic activity and for drug screenings. Here, we analyzed the susceptibility of *Helix* neurons to the epileptogenic drug PTZ, although other drugs could be tested. Indeed, previous studies have shown that drugs acting as anti-epileptic in humans demonstrated anti-epileptic functions in *Helix* neurons, such as lamotrigine, carbamazepine, levetiracetam, verapamil and valproate (reviewed in Altrup, 2004). The same phenomenon can be seen in other anticonvulsant drugs such as mephenesin, barbiturates, benzodiazepines and trimethadione that have been shown to be effective in invertebrate neurons (reviewed in Klee, 1976 and Altrup, 2004). Finally, a key advantage of this monosynaptic cell model is that the presynaptic and postsynaptic compartments can be selectively targeted by injections and specific networks can be constructed by directly plating individual neurons on MEA electrodes.

Conclusion

We showed that Syn-deficiency in *Helix* land snail neurons is associated with a lower threshold for the induction of epileptic activity. The increased cellular excitability is uncovered by treatments with the epileptogenic drug PTZ. Although the mechanism behind this action is

unknown, the characterization of the present monosynaptic cell model may help to develop future studies in this area, including epileptogenesis in Syn-depleted animal models, screening of new epileptogenic and antiepileptic drugs, and characterization of the mechanisms of action of anticonvulsant drugs at cellular and molecular levels.

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Author's contribution

The experiments included in this study were performed and the manuscript was mainly written by O.B.; S.G. participated in data acquisition; A.R. contributed to data analysis; and V.C. E.C., P.G.M. and M.G. participated in experiment planning, discussion and manuscript writing. All authors have read, corrected and approved the final version of the manuscript.

Conflict of Interest Statement

Authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations influencing the present article.

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Figure legends

Fig. 1. Synapsin knock-down increases cellular susceptibility to PTZ. (A) Sample recordings of the epileptic-like activity induced by PTZ in control and helSynKD neurons, horizontal bars represent the bath application of PTZ. Shown in detail are the spiking, action potential doublets and paroxysmal depolarization shifts (PDS) for one of the recordings. The susceptibility to PTZ was estimated by calculating the percentage of responding cells (B), the latency time (C) and the activity duration (D) of the control and helSynKD neurons. Data were analyzed with the chi-square and Kruskal-Wallis test, * $P < 0.05$, ** $P < 0.01$.

Fig. 2. Drug-induced epileptic-like activity characterization in single cells. The percentage of neurons showing spikes (A), action potential doublets (B) and paroxysmal depolarization shifts (PDS, C) was quantified during the treatments with PTZ at concentrations of 10, 20, and 40 mM. The total duration of spikes (D), doublets (E) and PDSs (F) was measured during the treatments with each PTZ concentration. Data were analyzed with the chi-square and Kruskal-Wallis test, * $P < 0.05$.

Fig. 3. Representation of the neuronal arrangement on MEA and sample epileptic-like recordings. (A) Scheme of neuron placement on a MEA, dark gray squares indicate the position of C1 neurons and light gray squares indicate the position of B2 neurons. (B) Phase contrast image of a C1-B2 pair and its growing neurites after 24 h of plating (left panel) and fluorescence image of a C1 neuron expressing EGFP reporter protein on MEA (right panel), scale bars: 100 μm. (C) Sample recording of PTZ-induced epileptic-like activity in Syn-silenced *Helix* neurons 48 h after plating, showing spiking activity, action potential doublets and paroxysmal depolarization shifts (PDS).

Fig. 4. Spontaneous and drug-induced epileptic-like activity on MEA. (A) Sample traces of PTZ-induced activity in control and helSynKD neurons on MEAs. Arrows indicate drug application. The percentage of presynaptic neurons exhibiting firing activity during PTZ application (B) or exhibiting spontaneous activity before drug perfusion (C) was determined at different time-points (24 h, 48 h, and 72 h after plating) in control and helSynKD neurons. Data were analyzed with the chi-square test.

Fig. 5. Drug-induced and electrical-induced epileptic-like activity characterization in MEA arrays during repetitive PTZ applications. The percentage of neurons showing spikes (A) and paroxysmal depolarization shifts (PDS, B) was quantified during the PTZ treatments 24 h, 48 h, and 72 h after plating. The duration of spikes (C) and PDSs (D) was measured during the treatments with PTZ at each time. The activity duration evoked by electrical stimulation was measured before drug application (Pretreatment, Pretr) and during drug application (PTZ) 24 h (D) and 48 h (E) after plating. Data were analyzed with the chi-square, the mixed ANOVA and the two-way ANOVA tests, *** $P < 0.001$.

Fig. 6. Synaptogenesis under recurrent drug-induced epileptic-like activity.
(A) Representative recording of spiking activity in a chemical connection between C1 and B2 neurons, cells are shown in the insert and the arrow indicates the direction of the synaptic connection (scale bar: 100 μm). (B) Sample cross-correlograms (CC), where 0 is represented as the gray dotted lines. (C) Delay between pre- and postsynaptic activity in control and synapsin-silenced neurons treated with PTZ. (D) Estimation of synaptic strength with the CC peak value in control and helSynKD neurons. (E) Occurrence of functional connections between C1 and B2 neurons in control and helSynKD pairs treated with PTZ at 24 h, 48 h, and 72 h. Data were analyzed with the Student's t-test.

Fig. 7. Neurite density of *Helix* neurons pairs cultured on MEA. (A) Bright field image of C1-B2 pair of control and synapsin-silenced neurons 48 h after plating, showing the neurites between paired cells. (B) The neurite density between C1 and B2 paired neurons growing on MEA devices was assessed in control and helSynKD neurons by counting the neurites crossing a fixed box of 200 μm^2 between the cells in MEA devices perfused with fresh medium (untreated) and in MEA devices perfused with 40 mM PTZ every 24 h (treated), neurite density is expressed in the 72 h time point for controls and helSynKD neurons treated (T) and not treated (NT). Data were analyzed with the two-way ANOVA and Kruskal-Wallis tests.

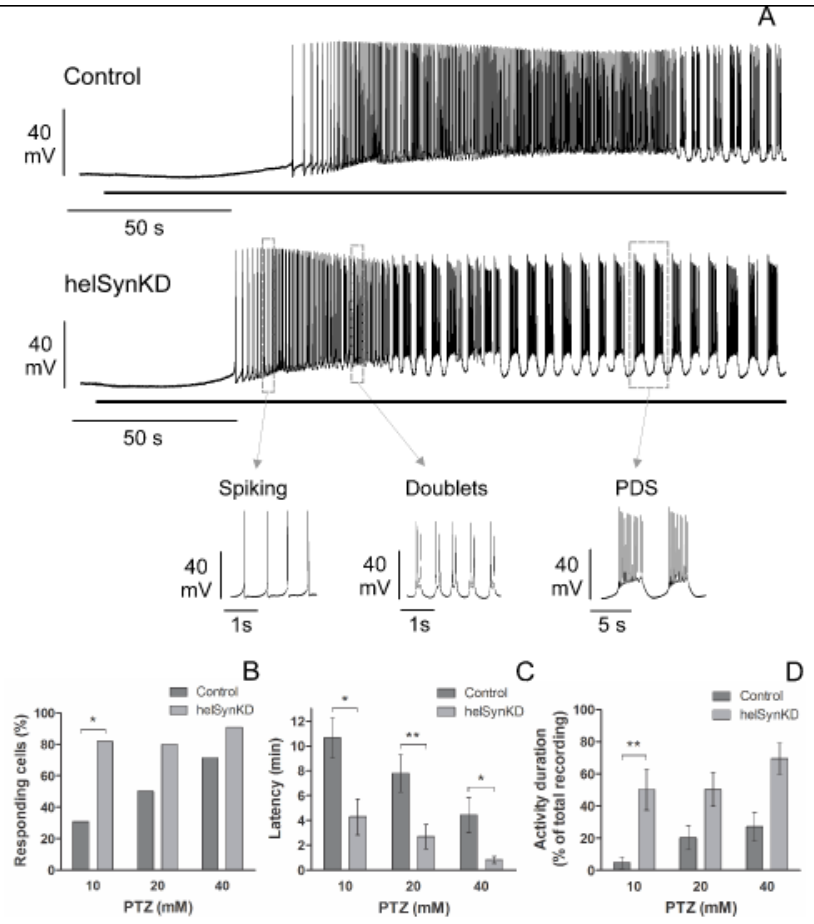

Fig. 1

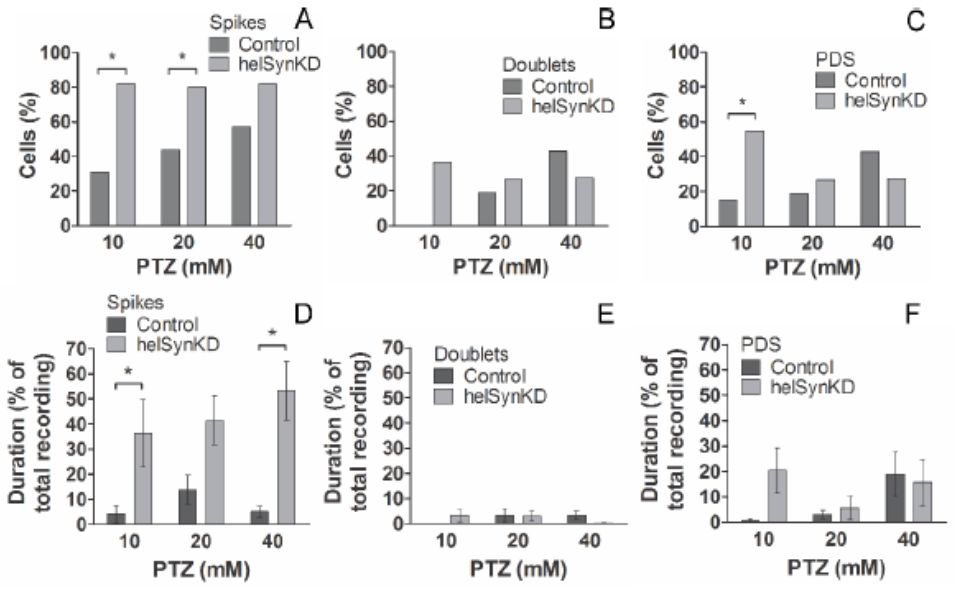
A
Spikes
Control
helSynKD
Cells (%)
PTZ (mM)
B
Doublets
Control
helSynKD
Cells (%)
PTZ (mM)
C
PDS
Control
helSynKD
Cells (%)
PTZ (mM)
D
Spikes
Control
helSynKD
Duration (% of total recording)
PTZ (mM)
E
Doublets
Control
helSynKD
Duration (% of total recording)
PTZ (mM)
F
PDS
Control
helSynKD
Duration (% of total recording)
PTZ (mM)

Fig. 2

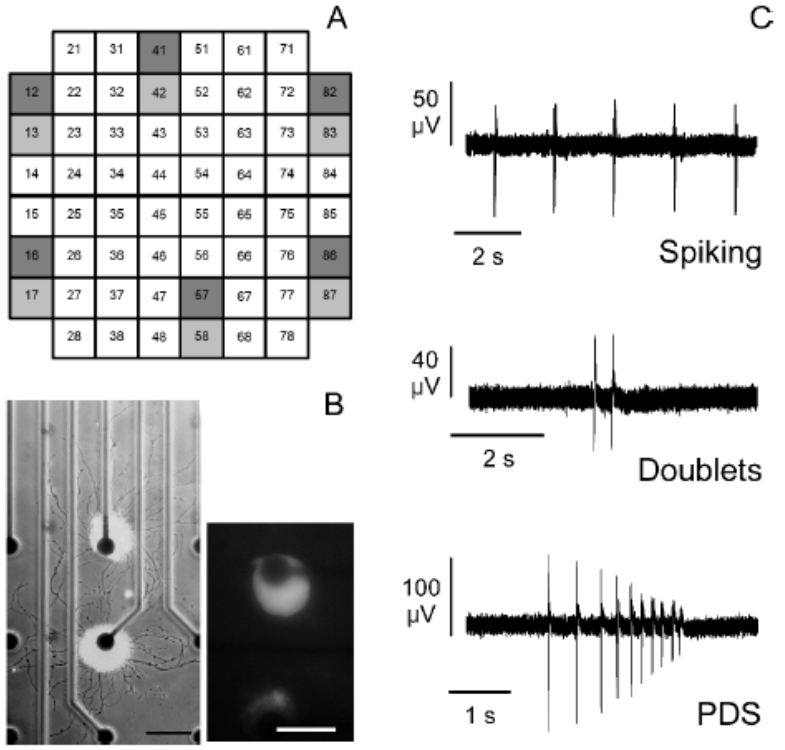
A
21 31 41 51 61 71
12 22 32 42 52 62 72 82
13 23 33 43 53 63 73 83
14 24 34 44 54 64 74 84
15 25 35 45 55 65 75 85
16 26 36 46 56 66 76 86
17 27 37 47 57 67 77 87
28 38 48 58 68 78
B
C
50 µV
2 s
Spiking
40 µV
2 s
Doublets
100 µV
1 s
PDS

Fig. 3

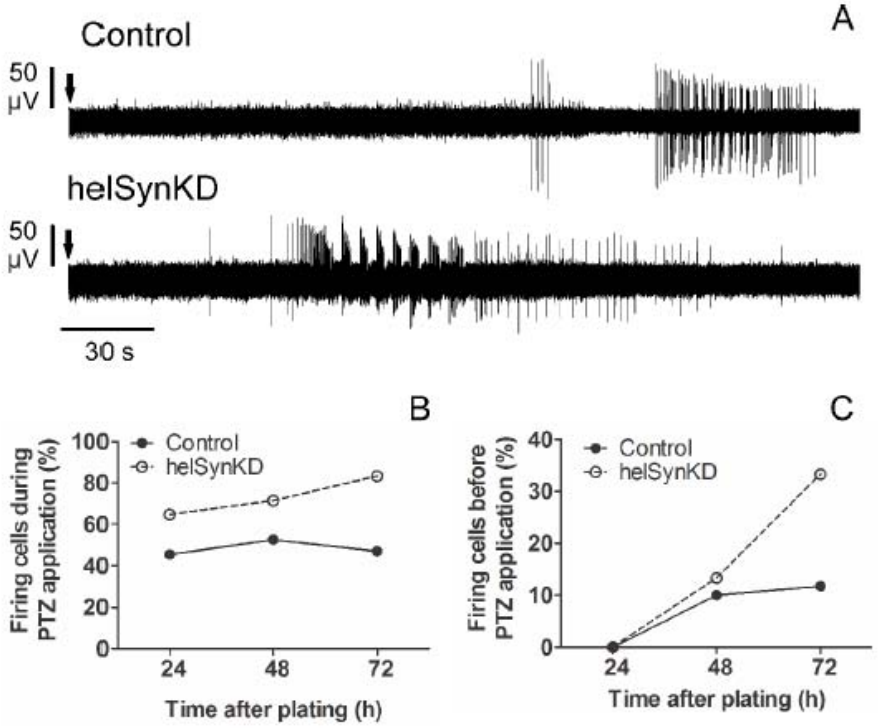
A
Control
50
μV
helSynKD
50
μV
30 s
B
Firing cells during PTZ application (%)
100
80
60
40
20
0
Control
helSynKD
24
48
72
Time after plating (h)
C
Firing cells before PTZ application (%)
40
30
20
10
0
Control
helSynKD
24
48
72
Time after plating (h)

Fig. 4

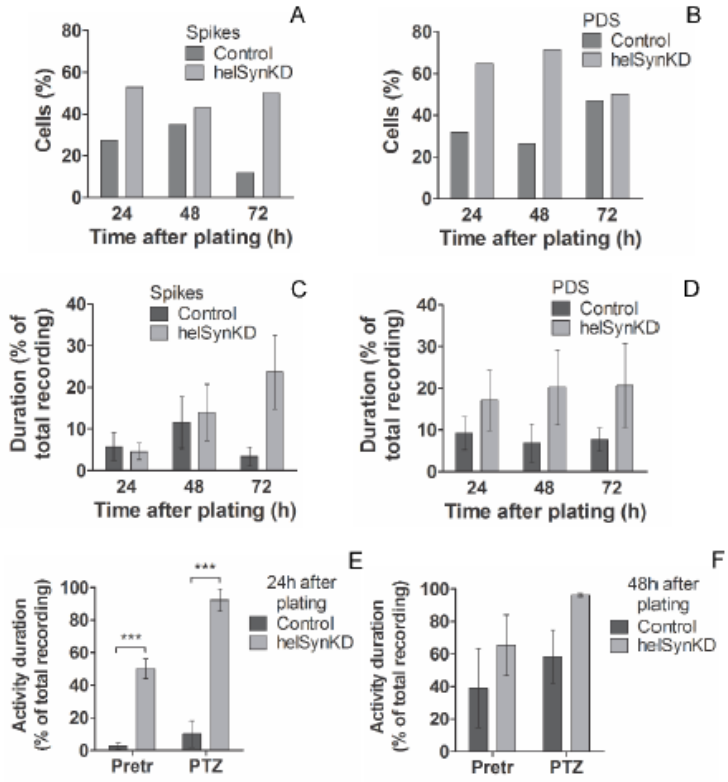
A
Spikes
Control
helSynKD
Cells (%)
Time after plating (h)
B
PDS
Control
helSynKD
Cells (%)
Time after plating (h)
C
Spikes
Control
helSynKD
Duration (% of total recording)
Time after plating (h)
D
PDS
Control
helSynKD
Duration (% of total recording)
Time after plating (h)
E
24h after plating
Control
helSynKD
Activity duration (% of total recording)
***
***
Pretr
PTZ
F
48h after plating
Control
helSynKD
Activity duration (% of total recording)
Pretr
PTZ

Fig. 5

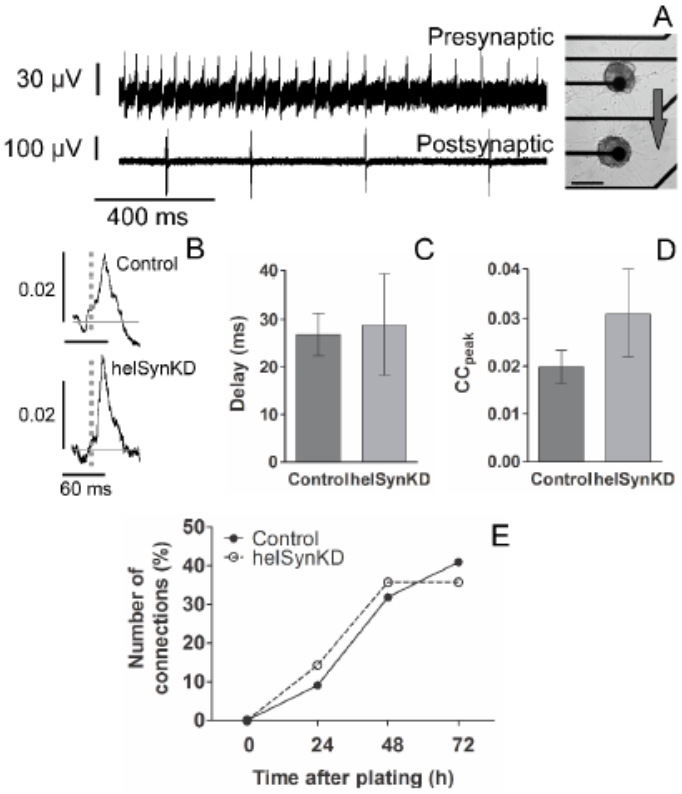
A
Presynaptic
30 µV
100 µV
Postsynaptic
400 ms
B
Control
0.02
helSynKD
0.02
60 ms
C
Delay (ms)
40
30
20
10
0
Control
helSynKD
D
CCpeak
0.04
0.03
0.02
0.01
0.00
Control
helSynKD
E
Control
helSynKD
Number of connections (%)
50
40
30
20
10
0
0
24
48
72
Time after plating (h)

Fig. 6

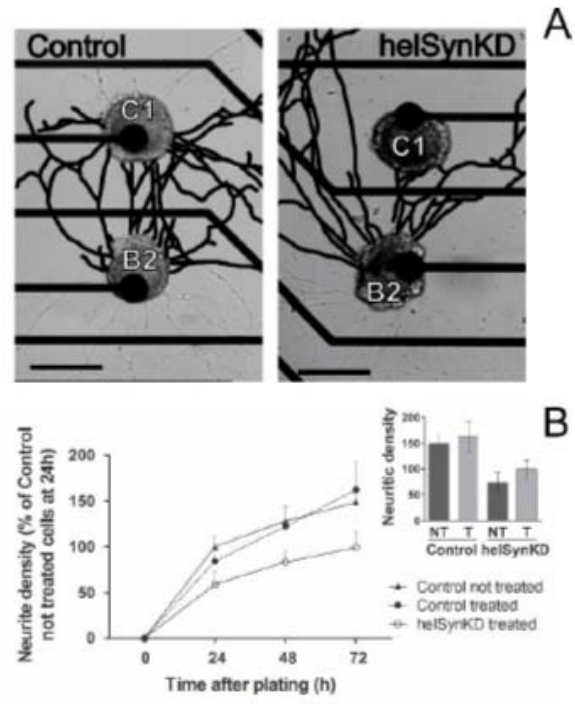
A
Control
helSynKD
C1
B2
C1
B2
B
Neurite density (% of Control not treated cells at 24h)
200
150
100
50
0
0
24
48
72
Time after plating (h)
Neuritic density
NT T NT T
Control helSynKD
Control not treated
Control treated
helSynKD treated

Fig. 7