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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.

1. 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

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.

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underlying drug-induced epileptic-like activity (Speckman and Caspers, 1973; Altrup and Speckmann, 1988; Madeja et al., 1989; Altrup et al., 1991, 2003, 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, 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, 2013) and intranuclear DNA injection (Brenes et al., 2015a,b). 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,b). 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.

2. Materials and methods

2.1. Materials

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

2.2. 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.

2.3. 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.

2.4. Plasmid generation and intranuclear microinjection

As previously described (Brenes et al., 2015a,b), 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,b).

2.5. Intracellular electrophysiological recordings

Standard intracellular recording techniques were used on single cultured cells as previously described (Fiumara et al., 2005, 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).

2.6. 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).

2.7. 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.