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Andrea Marcantoni[1,2,*], Elisabeth F. Raymond[1], Emilio Carbone[2], Hélène Marie[1,*]

Firing properties of entorhinal cortex neurons and early alterations in an Alzheimer's disease transgenic model.

[1] Institut de Pharmacologie Moléculaire et Cellulaire (IPMC), Centre National de la Recherche Scientifique (CNRS), Université de Nice Sophia Antipolis, UMR 7275, 06560 Valbonne, France.
[2] Department of Drug Science, Lab of Cellular & Molecular Neuroscience, University of Turin, Italy.

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* **Corresponding authors:**

Hélène Marie
Institut de Pharmacologie
Moléculaire et Cellulaire (IPMC)
CNRS, UNS, UMR 7275
660 Route des Lucioles
06560 Valbonne, France.
marie@ipmc.cnrs.fr

Andrea Marcantoni
Department of Drug Science and Technology
University of Torino
Corso Raffaello 30
10125 Torino, Italy
andrea.marcantoni@unito.it

Abstract

The entorhinal cortex (EC) is divided into medial (MEC) and lateral (LEC) anatomical areas and layer II neurons of these two regions project to granule cells of the dentate gyrus (DG) through the medial and lateral perforant pathway (MPP, LPP), respectively. Stellate cells (SCs) represent the main neurons constituting the MPP inputs, while fan cells (FCs) represent the main LPP inputs. Here, we first characterized the excitability properties of SCs and FCs in adult wild-type (WT) mouse brain. Our data indicate that during sustained depolarization, action potentials (APs) generated by SCs exhibit increased fast afterhyperpolarization (fAHP) and overshoot, making them able to fire at higher frequencies and to exhibit higher spike frequency adaptation (SFA) than FCs. Since the EC is one of the earliest brain regions affected during Alzheimer's disease (AD) progression, we compared SCs and FCs firing in 4 months old WT and transgenic Tg2576 mice, a well established AD mouse model. Tg2576-SCs displayed a slight increase in firing frequency during mild depolarization but otherwise normal excitability properties during higher stimulations. On the contrary, Tg2576-FCs exhibited a decreased firing frequency during mild and higher depolarizations, as well as an increased SFA. Our data identify the FCs as a neuronal population particularly sensitive to early pathological effects of chronic accumulation of APP-derived peptides, as occurs in Tg2576 mice. As FCs represent the major input of sensory information to the hippocampus during memory acquisition, early alterations in their excitability profile could significantly contribute to the onset of cognitive decline in AD.

Key words: action potentials, stellate cells, fan cells, Alzheimer's disease, Tg2576 mice

Introduction

Alzheimer's disease (AD) is the most common cause of progressive dementia in aging human populations, and an important medical, social and economic problem confronting contemporary society. It is characterized by progressive memory loss involving dysfunction of the medial temporal lobe (MTL), including the hippocampus and the entorhinal cortex (EC) [40]. A large body of evidence supports the "amyloid-cascade hypothesis", which posits that accumulation of amyloid-β (Aβ) into oligomers is the toxic initiating event triggering the progressive dismantling of synaptic communication, as well as perturbations in neuronal network excitability in these memory encoding areas [31, 36]. Two forms of AD exist: a familial AD (FAD) accounting for about 1% percent of AD cases and a sporadic AD. Most FAD mutations occur on the amyloid precursor protein (APP) or on proteins involved in its processing (presenilin 1 and 2), resulting in early pathological accumulation of Aβ in the MTL. An identical Aβ accumulation is observed in sporadic AD but its cause is not clear [4, 45].

The EC represents the main source of afferent inputs to the hippocampus [20]. Within the MTL, the EC is an initial target in AD as significant loss of neurons in EC layer II occurs in the early stages of the disease [16, 42]. Structural and functional imaging studies also show early, selective atrophy and hypometabolism in the EC of patients with mild cognitive impairment or early-stage AD [29, 46, 56]. The EC is divided into the medial (MEC) and lateral EC (LEC). There is evidence in rodents and humans that these two EC sub-regions mediate different spatiotemporal and sensory inputs to the hippocampus, necessary to form episodic memories [7, 12, 35, 55]. Cells within the layer II of the EC heavily innervate the dentate gyrus (DG) with fibres originating from the MEC terminating in the middle third of DG molecular layer, thus forming the medial perforant path (MPP); and fibres originating from the LEC terminating in the distal one-third of the DG molecular layer, thus forming the lateral perforant path (LPP). Within the layer II of the MEC, the most abundant neurons are represented by the stellate cells (SCs), which form the MPP [1]. Within the layer II of the LEC, the most abundant neurons belong to another type of stellate cells, the so-called

"fan cells" (FCs), which form the LPP [43]. Both SCs and FCs are morphologically similar, characterized by multiple radiating dendrites that spread over layers II and I [43]. Functionally, however, these two cell types have been characterized in rat brain and exhibit specific differences. SCs are characterized by a pronounced time-dependent inward rectification current, mainly due to the activity of a hyperpolarization-activated inward current (I_h), and by persistent sub-threshold oscillations, activity that corresponds to non-periodic fluctuations, both of which are less pronounced in FCs [1, 10, 43].

While several electrophysiological studies have been performed on transgenic AD mouse models to study the nature of Aβ-induced synaptic and excitability defects within the hippocampus [28, 31, 33], nothing is known about the Aβ-induced alterations in neuronal excitability in EC neurons. Here, for the first time, we investigated the integrity of the intrinsic excitability of SCs and FCs in a well-known AD mouse model, the Tg2576 mice [18]. These mice harbour the human APP gene with the Swedish mutation and display age-dependent accumulation of Aβ within the MTL [11, 18, 44]. We previously identified early hippocampus-dependent contextual memory deficits in Tg2576 mice older than three months [9]. We now asked if these early symptomatic mice display alterations in the excitability profile of SCs and FCs of the EC. To address this issue, we first characterized the excitability profile of these neurons in 4 months old wild-type (WT) mice. We observed that when stimulated by injecting minimum amount of current necessary to induce AP firing, SCs fired at higher frequencies than FCs, as a consequence of their ability to generate APs with higher fAHP and overshoot. Similarly, when SCs were maximally stimulated by injecting larger amounts of currents, they fired at higher rates and exhibited increased spike frequency adaptation (SFA), probably because of the increased contribution of both I_h and Ca^{2+}-activated K^+ channels (BK and SK channels). We also compared the excitability properties of SCs and FCs in 4 months old WT mice and Tg2576 mice. We observed that, while SC excitability was only mildly affected in transgenic mice, FCs of Tg2576 mice exhibited a significant decrease in AP firing frequency and an increased SFA. Our experiments thus reveal that, in adult mice as in rats, the electrophysiological

properties of SCs and FCs, critical for understanding the physiological function of these neurons, are significantly different. We also conclude that FC excitability is significantly altered in Tg2576 mice, suggesting possible correlations with early Aβ accumulation and onset of memory impairment.

Material and Methods

Ethical approval:

All experiments were done according to national policies on the care and use of laboratory animals in accordance with the European Communities Council Directive (86/609/EEC). All efforts were made to minimize animal suffering and reduce the number of animals used. The local competent French committee approved the experiments (authorization number A06-264).

Mice

Hemizygous Tg2576 mice (n= 10) carrying the human Swedish mutation (APP695 with double mutations at KM670/671NL) [18] and wild-type (WT, n=13) littermates were genotyped and used for the experiments as previously described [9]. All experiments were done with interleaving WT and Tg2576 mice between experimental days. Also SCs an FCs derived recordings were regularly alternated during the same experimental day. The animals were housed under controlled laboratory conditions with a 12 h dark light cycle, a temperature of 21 ± 2°C, and a humidity of 60-70%. Mice had free access to standard rodent diet and tap water.

Slice preparation

Mice were killed with halothane gas anaesthesia and brains were quickly removed. 250 µm thick transverse slices containing the entorhinal cortex and hippocampus were dissected out, according to the method described by Leutgeb et al. [26]. In order to limit the dorso-ventral location of slices and

relative changes in neuronal electrophysiological properties [14, 32], we started to collect horizontal slices from the midline part of the entorhinal cortex and then gradually reached the dorsal side. Slices containing both the LEC and MEC [26, 55] were cut and collected in ice-cold cutting solution containing (in mM): 2.5 KCl, 1.25 NaH2PO4, 10 MgSO4, 0.5 CaCl2, 26 NaHCO3, 234 Sucrose, and 11 Glucose (saturated with 95% O2 and 5% CO2, pH= 7.10). After the dissection, slices were kept in oxygenated artificial Cerebro-Spinal Fluid (ACSF) made of (in mM) 119 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 26 NaHCO3, and 11 Glucose, at a temperature of 36°C for 1 h, and then at room temperature (RT). By varying the temperature from RT to 36°C the pH of the ACSF increased from 7.30 to 7.39. The pH was not adjusted during the experiments.

Electrophysiological recordings

Electrophysiology set up

Slices were placed in a submerged recording chamber at 30–32 °C in oxygenated ACSF and visualized under IR-DIC on a slicescope at x60 magnification (Scientifica LTD, UK). Recordings were made using a patchstar micromanipulator (Scientifica LTD, UK) connected to a Mutliclamp 700B amplifier, Digidata 1440 acquisition system and pClamp 10 software (Axon instruments, Molecular Devices Ltd, USA). Patch pipettes were made of borosilicate glass and shaped to a final resistance of 7-10 MΩ.

Cells identification

Neuron identification as SCs from layer II of the MEC or FCs of layer II of the LEC was performed by considering their different localization [5, 6, 7, 55], and their morphological similarities [43]. First, we identified SCs and FCs by localizing them in the upper part of layer II, while pyramidal cells were located deeper within this layer. (Hippocampus. 1997;7(5):571-83.”Morphological characteristics of layer II projection neurons in the rat medial entorhinal cortex”.Klink R, Alonso A.). Moreover, both SCs and FCs were characterized by the presence of two or more major

dendrites running over layer I [1], as well as by ovoid rather than triangular and larger soma [53]. Upon patching the neurons, the final confirmation of their neuronal typology was obtained by analyzing their electrophysiological properties, including the presence of subthreshold voltage oscillations, the ability to fire clusters of APs, the ability to generate an inward rectification potential during hyperpolarizing current pulses (higher in SCs than in FCs) and a pronounced depolarizing after-potential (DAP) (Fig.1H) [10]. Cells without these AP kinetics, probably representing pyramidal neurons [1, 23], were not further considered for recordings or analysis. We did not detect differences in the number of available and healthy neurons between WT and Tg2576 slices at this age..

Current clamp experiments

Whole-cell patch clamp experiments were performed in the current clamp configuration using a pipette solution containing (in mM): 135 gluconic acid (potassium salt: K-gluconate), 5 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, 0.4 Tris-GTP. Unless otherwise stated, appropriate electrode capacitance and off-line bridge compensation was applied. After a tight seal (>1 GΩ) on the cell body of the selected neuron was obtained, whole-cell patch clamp configuration was established and cells were left to stabilize for 2-3 min before recordings began. The resting (V_m) membrane potential was first measured in the absence of any spontaneous firing and only cells with V_m more negative than -55mV were considered. We then injected a minimum amount of current (200 pA) to stimulate a sustained firing that we recorded for few minutes. Using this tonic firing, we measured the fast and medium afterhyperpolarization potentials (fAHP and mAHP, respectively) [41] (Fig.1G), as well as the depolarizing after-potential (DAP) defined as the difference between the after depolarization potential (ADP) and fAHP [1]. The ADP amplitude corresponds to the depolarizing peak that follows the fAHP and determines the formation of the typical “sag”. The maximum rising slope, the overshoot and the action potential half-width were also considered (Fig.1G). The half-width value was calculated considering the AP width measured

at 50% of the peak amplitude. These AP parameters were estimated without taking into account the voltage drop across the pipette resistance. We estimated that for 200 pA of injected current, the mean voltage error introduced by the pipette was 2.1 ± 0.1 mV. The coefficient of variation (CV) of the interspike interval (ISI) was measured by dividing the average value of standard deviation by the average value of instantaneous firing frequency. The membrane resistance was obtained by clamping neurons at V_h= -60 mV, injecting -200 pA of current, measuring the amount of steady-state hyperpolarization and calculating the resistance value by Ohm's law. The rheobase was estimated by measuring the minimum amount of current necessary to induce AP firing from V_h= -60mV.

To study the relationship between firing frequency and current input (Fig. 2A, B), we first adjusted the membrane potential (V_h) to -60mV and then injected 10 pulses of increasing intensity (from 300 to 550 pA, 200 ms duration). We estimated that for injections of 550 pA, corresponding to the maximum amount of current injected to induce repetitive firing, the pipette resistance introduced an average error of 5.6 ± 0.2 mV that should be subtracted from membrane potential values. We also used these recordings to measure the instantaneous firing frequency at the beginning (onset frequency, $f_{o,}$ corresponding to the firing frequency measured between the first and second AP in the spike train) and at the end of the spike train (Fig. 2A) (steady state frequency, f_{ss}, corresponding to the firing frequency measured between the last two APs in the spike train). By plotting f_o and f_{ss} as a function of injected current (Fig. 2C, D), we obtained information on the spike frequency adaptation of these neurons [3].

The involvement of Na^+ channels in spike generation was derived from the "phase-plane" plot obtained by drawing the first time derivative of voltage (dV/dt) versus voltage [2, 19, 48] (Fig.3B). We could thus estimate the amount of transient Na^+ current contributing to the spike up-stroke from the maximal value of dV/dt (dV/dt_{max}) (Fig. 3A) [8, 15] and the spike threshold as the voltage at which dV/dt was 4% of dV/dt_{max} (Fig.3C) [21]. We restricted the analysis of Na^+ channels

availability in spike generation by considering only the APs generated after injection of 550 pA, a current step inducing the greatest firing frequency difference between SCs and FCs (Fig. 2B).

To quantify the inward rectification time-dependent potential [10, 51], we first adjusted the membrane potential (V_h) to -60mV and injected 10 pulses of increasing intensity (from -100 pA to -1 nA, 600 ms duration). During the pulse, we observed that the hyperpolarization reached a maximum value (peak) and then decreased to stabilize to a steady-state value (Fig. 4A, B). We plotted the peak and the steady state values as a function of injected current to obtain indirect information on the hyperpolarization-activated inward current (I_h). The activation time of the inward rectification potential was estimated by fitting the curve with an exponential algorithm and measuring the time constant (τ_{on}) (Fig. $4A_1$).

We measured the fAHP and AHP of each AP within the spike train generated by injecting 550 pA of current and plotted them (Fig. 4C, D, F), as well as the AP half-width (Fig.4E) as a function of the number of APs in the spikes train. Both the fAHP and half-width parameters gave indirect information about the ion channel conductances responsible for the generation of consecutive APs. About the AHP, it was generated by depolarizing stimuli represented by 20 pulses of current of 200 ms duration and increasing intensity (from 100 to 1050 pA; Fig. 4C, F), considering that this parameter is largely modulated by Ca^{2+}-activated K^+ conductances [27].

Analysis and statistics

Data analysis were performed using either Clampfit version 10.0 (Molecular Devices) or Origin Pro 6.0 (OriginLab). Data are given as mean ± SEM for the number (*n*) of cells. Statistical significance (*P*) was calculated using the unpaired two-tailed Student's *t* test. Data were considered statistically significant with $P< 0.05$.

Results

Comparison of passive membrane and AP properties of SCs and FCs in adult WT mice

SCs (n= 19) and FCs (n= 17) from layer II of the MEC and LEC, respectively (Fig. 1A), exhibit nearly identical resting membrane potential (V_m) (see Table 1), and low R_m (<100 MΩ), which turned out to be significantly lower in SCs compared to FCs (Table 1; first two rows). EC neurons were not able to generate spontaneous APs at rest (Fig. 1B, C), but 200 pA of current injection was sufficient to achieve membrane voltages between -50 and -45 mV (from V_m of ~ -65 mV) and to induce slow repetitive firings in both cell types (Fig. 1B, C). Comparison of the average interspike interval (ISI) and their coefficient of variation (CV) revealed that both ISI and CV increased respectively by 28% and 35% (**P< 0.01) in FCs (Fig. 1D-F), thus unmasking the tendency of FCs to fire at lower rate and more irregularly, despite they had higher R_m than SCs. The average firing frequencies estimated by the average ISI were respectively 1.25 Hz for SCs and 0.98 Hz for FCs. As previously reported in rat brain slices [32, 43], both neurons generate clustered patterns of APs especially when minimum amounts of currents were injected to induce the firing (~200 pA) (Fig. 1B, C).

By comparing the AP properties (Fig.1G) of both cell types (Fig. 1H), we observed that the fAHP of SCs was significantly increased with respect to FC neurons (*P< 0.05) (Table 1; Fig.1H), suggesting higher contribution of repolarizing K^+ conductances in SCs than in FCs. This difference is also reflected in the larger DAP observed in SCs compared to FCs (*P< 0.05) (Table 1; Fig. 1H). Finally, the AP overshoot was significantly higher in SCs compared to FCs (*P< 0.05), most likely as a consequence of the more negative potentials reached by SCs during the fAHP. The increased fAHP confers to SC neurons the ability to recover higher densities of Na^+ channels from fast inactivation during trains of spikes (Table 1; Fig. 1H). We thus conclude that long lasting depolarizations of 200 pA enable SCs to fire at higher frequencies and more regularly than FCs and that this property derives from the ability of SCs to exhibit APs with more pronounced fAHPs and higher overshoots.

Analysis of firing behavior in SCs and FCs of adult WT mice

The firing of the two population of neurons was further analyzed by injecting current steps from 300 to 550 pA (200 ms duration), while holding the membrane potential (V_h) at -60 mV (Fig. 2A). We first observed that the rheobase value (Fig. $2B_1$) was significantly lower in FCs (*P< 0.05) despite the higher R_m and that during step depolarizations, the firing frequency of both cells increased markedly with respect to the firing near rest (from 1 to ~20 Hz) (Fig. 2B). The firing frequency of the two cell types was identical up to 450 pA and started to increase in SCs above 450 pA. At 550 pA SCs (n= 24) had mean maximal frequency of 54.3 Hz compared to 48.3 Hz in FCs (n= 23; *P< 0.05) (Fig. 2B).

To better evaluate the ability of these two populations of neurons to adapt their firing behavior with time [3], we measured the instantaneous firing frequency at the beginning (onset instantaneous firing frequency, f_o) and at the end of the current steps (steady-state instantaneous firing frequency, f_{ss}) during current steps of 300-550 pA (Fig. 2A). We observed that f_o was consistently higher in SCs compared to FCs (Fig. 2A and C) (*P< 0.05, **P< 0.01), while f_{ss} was nearly similar and achieved maximal values in both cell types, indicating that pulses of 200 ms were sufficient to estimate f_{ss} (Fig. 2D). Indeed, when using longer pulses (550 pA for 4 s) f_{ss} drastically decreased probably as a consequence of the increased activation of Ca^{2+}-activated K^+ conductance, but did not change significantly between the two cell types (10.3±3.7 Hz, n= 8 in SCs vs 13.8±1.2 Hz, n= 8 in FCs). These results suggest an increased SFA in SCs with respect to FCs, which derives from the higher f_o (Fig. 2B). We next indirectly analyzed the properties of the main ion channels involved in AP firing in these neurons.

Involvement of sodium channels in setting the firing frequency in SCs and FCs of adult WT mice

Additional analysis of AP shape was performed to investigate the involvement of Na^+ channels in setting the firing frequency of these two populations of neurons. Using phase plane plots of dV/dt vs. V for the first and last AP of trains generated during 550 pA current steps (Fig. 3B), we estimated the average dV/dt_{max} as an indication of the maximal Na^+ currents passing during an AP [8, 15], and the voltage at which dV/dt is 4% of its maximum as the threshold potential of AP

generation [21, 48]. As shown in Fig. 3A-C, the mean dV/dt_{max} and spike threshold values of the first and last APs were similar in both cell types (n= 21 SCs; n= 22 FCs). We therefore conclude that the Na^+ channels responsible for AP generation are not the main causes of the different firing behavior observed in these neurons (Fig. 2B). Interestingly, we found that the shape of the phase plane plot was rather regular in all cells and exhibited no clear sign of "kink" associated to the AP generated in the initial segment that precedes the somatic spike [2]. This suggests that our recordings mainly reflect somatic spikes occurring at comparable distances from the initial segment where the spike is generated.

Involvement of the hyperpolarization-activated inward current (I_h) in setting the firing frequency in SCs and FCs of adult WT mice

It is known that both SCs and FCs of the EC display a time-dependent inward rectification in the hyperpolarizing direction associated with the activation of the I_h current [1, 10]. It is also known that in rat EC, the size of I_h in SCs is higher than in FCs [43, 51]. In addition, I_h contributes to set the resting potential around -60 mV [10], and increases during sustained step hyperpolarizations to reach steady-state values. Our experiments confirmed that in adult WT mice, as in rat, the I_h-driven inward rectification in SCs is more pronounced than in FCs and leads to lower steady-state hyperpolarization levels [43, 51]. On average, with hyperpolarizing pulses of -500 pA, the newly activated I_h developed faster in SCs (τ_{on} 58.0±5.0 ms, n= 22) with respect to FCs (97.1±12.2 ms; $^{**}P<0.01$, n= 21) (Fig. $4A_1$) and caused more pronounced depolarizations at the end of the pulse which reached maximal values with current steps of -1 nA (V_m= -54.5 ±2.5 mV in SCs vs. -68.6±4.3 mV in FCs; $^{**}P< 0.01$) (Fig. 4B). The presence of a larger I_h in SCs was also evident on return to -60 mV following prolonged hyperpolarization. The I_h in fact caused a more rapid membrane depolarization followed by one or more APs (Fig. 4A), which were observed in 70% of SCs and only 36% of FCs. Since I_h is an unspecific inward current carried mainly by Na^+ and K^+ ions that generates slow and non-inactivating membrane depolarizations, we suggest that a higher contribution of I_h to the slow interspike depolarization could account for the increased firing

frequency of SCs [39]. We cannot also exclude, however, a contribution of T-type calcium channels effectively recruited during prolonged hyperpolarization that help accelerating the following rapid depolarization [Carbone & Lux, 1987; Kinetics and selectivity of a low-voltage-activated calcium current in chick and rat sensory neurones, J Physiology, 386; 547-570].

AHP properties in SCs and FCs of adult WT mice

To understand if the firing properties of SCs and FCs are regulated by different mechanisms of AP repolarization, we compared the amplitude of the fAHP of each AP during spike trains generated by 550 pA current steps (Fig. 4D) and the AHP following sustained depolarizing stimuli of increased amplitude (Fig. 4F). This latter is known to be associated to the activation of a Ca^{2+}-activated K^+ conductance [27] that is critical for the control of firing rate [34]. We found that both the fAHP and AHP were significantly higher in SCs. In particular we noticed that fAHP increased in SCs at the beginning of the spike train (1^{st} and 2^{nd} AP; ***P< 0.001 for the 1^{st} AP and **P< 0.01 for the 2^{nd} AP), while became comparable from the 3^{rd} AP onwards in both SCs (n= 20) and FCs (n= 19) (Fig. 4D). The AHP increased proportionally to the amplitude of the depolarizing pulse to reach maximal values above 800 pA. At these maximum values, the AHP was significantly higher in SCs with respect to FCs (*P< 0.05). On average, we estimated maximal differences of 2.9 mV with current steps of 1050 pA (Fig. 4F). This suggests higher contribution of Ca^{2+}-activated K^+ channels in setting the higher firing rate and SFA of SCs. We also measured the half-width of the APs and found no significant difference between the two cell types (Fig. 4E).

Comparison of SC firing properties of the MEC in WT and Tg2576 mice

While characterizing the properties of SCs and FCs in WT mice, we also recorded the activity of these cells in four months old early symptomatic Tg2576 littermates to identify possible early excitability alterations. At this age, Tg2576 mice display early accumulation of Aβ in the MTL and begin to show deficits in contextual memory [9, 11, 18, 44]. We found that the excitability properties of SCs of Tg2576 mice (n= 18) were only slightly different from SCs of WT mice (n= 19). Neither the passive membrane properties (R_m and V_m), nor the other AP parameters were

altered during sustained firings (Table 1). A slight, but significant decrease in the fAHP was, however, noted in SCs of Tg2576 mice ($^{\#}P< 0.05$, Table 1), suggesting alteration of K^+ conductances in SCs of these mice. Surprisingly, the average ISI was nearly halved in Tg2576-SCs (***$P< 0.001$; Fig. 5E) and the mean firing frequencies obtained from the ISI values were respectively 1.3 Hz for WT-SCs and 2.3 Hz for Tg2576-SCs. This suggests a tendency of Tg2576-SCs to fire at higher rates near rest, although maintaining a high-degree of irregularity, as indicated by the high CV of the ISI distribution (Fig. 5C, D).

While analyzing the firing behavior with increasing input stimuli, we observed that both the rheobase values (Fig. $5G_1$), the spike frequency (Fig. 5F, G) and the SFA of Tg2576-SCs (n= 24) were comparable to the WT-SCs (n= 24) (Fig. 5H, I). We also tested if longer pulses (4 s) of 550 pA were able to unmask an altered SFA of Tg2576-SCs, but we did not observe any significant difference in the f_{ss} value (10.3±3.7 Hz; n= 8 in WT-SCs vs 12.8±3.7 Hz, n= 9 in Tg2576-SCs). Na^+ channel involvement and threshold of AP firing during the first and last AP were also unchanged, as estimated by the dV/dt_{max} and 4% dV/dt_{max} values (Fig. 5L, M). Finally, the membrane hyperpolarization properties (peak and steady-state), involving the I_h current, were similar to WT (Fig. 6A). The same was true for the fAHP within the spike train, the AP half-width and the post-train AHP (Fig. 6B-D). The amplitude of AHP remained nearly unchanged when longer depolarizing current pulses (550 pA for 4s) were used (-11.6±0.7mV; n= 8 in WT-SCs vs -13.2±1.0 mV; n= 9 in Tg2576-SCs). We therefore conclude that the excitability properties of SCs in Tg2576 mice, forming the main input to the DG through the MPP, are only partially perturbed during the early phase of AD. The only observed alterations were the reduced fAHP and the ISI during mildly stimulations, possibly due to a decrease of K^+ channels activation necessary for cell repolarization. Apart from this, the overall excitability profile of SCs appears mostly unaltered in these early symptomatic Tg2576 mice.

Comparison of FC firing properties of the LEC in WT and Tg2576 mice

A similar comparison was performed between FCs of 4 months old WT (n= 17) and Tg2576 (n= 18) littermates. We could not detect any significant difference in the passive membrane properties and AP kinetics during tonic firing (Table 1). During sustained firing with minimal current injection, however, we found that the average ISI increased by 25% in Tg2576-FCs (**P< 0.01) (Fig. 7E), preserving a high degree of irregularity as indicated by the high CV of the ISI distribution in both WT-FCs and Tg2576-FCs (Fig. 7C, D). The related mean firing frequencies were respectively 0.98 Hz for WT-FCs and 0.78 Hz for Tg2576-FCs.

When stimulated with increasing squared current pulses, the rheobase of FCs in Tg2576 mice was consistently higher than in WT (Fig. 7F, G_1) while the average firing frequency decreased regardless of R_m, which was unchanged (Fig. 7G; Table 1). On average, 550 pA current injections induced spike trains of 4.6 Hz lower frequency in Tg2576-FCs (n= 21) with respect to WT-FCs (n= 24). Also, the analysis of firing adaptation revealed that the lower frequency of Tg2576-FCs was mainly due to a lowered f_{ss} while f_o remained nearly unaltered regardless of current injection (Fig. 7H, I). All together these results reveal that SFA is increased in Tg2576-FCs.

Concerning the dV/dt_{max} and the voltage of AP threshold (4% dV/dt_{max}) associated to voltage-gated Na^+ channels (Fig. 7L,M), the membrane hyperpolarization properties, associated with the I_h current (Fig. 8A) and the post-train AHP values mainly related to Ca^{2+}-activated K^+ channels (Fig. 8B,E), they were all found not significantly different. The AHP was also found no significantly different after injection of long lasting (4 s) current steps of 550 pA (-11.3±0.5 mV; n= 8 in WT-FCs vs -8.7±0.1 mV; n= 11 in Tg2576-FCs). On the contrary, we found that the APs of Tg2576-FCs (n= 21) had significantly greater fAHPs (Fig. 8B, C) and shorter half-width compared to WT-FCs (n= 24) (Fig. 8B,D; *P< 0.05, **P< 0.01). Specifically, the fAHP increased from the 3rd AP onwards (Fig. 8C) and reached maximal difference of 3.2 mV during the 7th AP. The half-width were smaller for all APs of the train in Tg2576-FCs (Fig. 8D) and maximal difference of 0.2 ms was reached during the last AP. This suggests an increased contribution of the repolarizing conductance in Tg2756-FCs, probably due to an increased availability of voltage-gated K^+ channels. We conclude that, while the

passive membrane properties and AP parameters are unaffected in Tg2576-FCs during sustained firing, these neurons exhibit lower firing frequencies with an otherwise similar high degree of irregularity. Robust stimulation of FCs to generate high-frequency trains of APs unmasked a significant increase in SFA of Tg2576-FCs compared to WT-FCs, resulting in a lower rate of firing. Our results thus demonstrate that early symptomatic Tg2576 mice exhibit specific alterations in LEC function due to a reduced firing rate of FCs.

Discussion

In this study, we first described the excitability profile of entorhinal cortex SCs and FCs in WT adult mice as, to our knowledge, previous reports on these cells where limited to rat brain slices [5, 6, 43]. We provide clear evidence that differences in AP waveform between SCs and FCs in adult mice determine the differences in firing regularity and frequency, both of them significantly increased in SCs. In good agreement with data from rat brain slices [37], we found that the rheobase of FCs is significantly lowered, mainly because of the higher resting membrane resistance, indicating that these neurons are more prone to repetitive firing than SCs. On the other hand, during maximal current injection, SCs initially fire at higher frequency than FCs, but adapt more rapidly to steady-state frequencies. Since a rapid neuronal adaptation is correlated with the ability of neurons to synchronize with the surrounding network [13, 25], it is likely that, the increased SFA of SCs may enhance the effectiveness of their synaptic input on the granule cells of DG.

We also attempted an indirect analysis of the ionic currents responsible for the higher firing frequency and faster adaptation properties in SCs. Based on the analysis of the dV/dt_{max} amplitude, we found that during AP trains, Na^+ channel availability in SCs is comparable to FCs (Fig.3). On the contrary, we found that the time-dependent inward rectification underlying the contribution of I_h is significantly higher in SCs than in FCs (Fig. 4A, B). A larger I_h in SCs has been already reported in adult rat [10, 43] but it is here indirectly described for the first time in mouse. As I_h is known to control the frequency of repetitive firing [39], the larger inward rectification observed during

prolonged hyperpolarizations in SCs (Fig.4A) could contribute to increase spike frequency and adaptation of SCs.

Another main difference between SCs and FCs firings concerns the fAHP of single spikes. In SCs, the fAHP during the firsts APs was significantly higher than in FCs, suggesting a stronger contribution of K^+ currents during the early repolarization phase of the train (first two APs). This current, however, inactivates during subsequent APs and is unable to affect the AP duration as indicated by their unchanged half-width. It is interesting to notice that this increased fAHP of SCs is most likely responsible for the increased recruitment of I_h, which probably represents the main source of inward current sustaining the higher instantaneous frequency (f_o) of spike trains.

A second major difference was found on the size of the AHP that follows prolonged repetitive firings and drives the final repolarization on return to rest. SCs displayed more pronounced post-train AHPs with respect to FCs (Fig. 4F). Channels underlying an M-current might play a role in the spike accommodation process [27], but its relevance in these neurons is still to be confirmed [17, 32]. On the other hand, the Ca^{2+}-dependence of AHP [27] and its presence in SCs has already been documented [22]. The underlying Ca^{2+}-dependent K^+ currents, usually carried by the small-conductance SK channels, are responsible for the slowing down of AP firing driven by the amount of Ca^{2+} accumulating into the cells during an AP train. It is possible that in EC neurons, like in other neurons and neuroendocrine cells [48], SK channels contribute to set the frequency of irregularly firing cells and the spike frequency adaptation during sustained depolarization.

SCs and FCs firings are differently altered in early symptomatic TG2576 mice

An interesting finding of our work is that the excitability properties of SCs and FCs are differently affected during the early symptomatic phase of the pathology and the onset of cognitive deficits in Tg2576 mice. SCs are little altered while FCs are more significantly modified. In Tg2576-SCs only the fAHP during sustained tonic firing was significantly increased, suggesting slight impact of early AD onset on the underlying K^+ conductances. This mild alteration was however sufficient to decrease the ISI in Tg2576-SCs during minimal depolarization near rest (Fig. 5). On the contrary,

there were no differences between WT and Tg2576-SCs during maximal stimulation (Fig. 5). This suggests that at the early symptomatic phase, SCs altered functionality applies only when neurons are stimulated near resting conditions, while preserving the ability of the EC to convey spatial stimuli to the hippocampus necessary for memory formation [55].

At variance with SCs, the firing frequency and the rheobase of Tg2576-FCs were both significantly smaller than WT-FCs (Fig. 7). A reduced frequency near resting conditions and during sustained stimulation, indicates a change in a large dynamic range of FCs firing. This is also accompanied by an increased SFA and led to significant lowered firing rates toward the end of AP trains. The most reasonable explanation for this is related to the increased fAHPs and decreased AP duration, suggested by the smaller AP half-width. At variance with this, there was no change of the maximal dV/dt associated with the availability of fast inactivating Na^+ channels and of inward rectification during hyperpolarizing pulses associated with I_h. All this indicates that FCs of early symptomatic TG2576 mice are probably characterized by an enhanced K^+ conductance, operated by voltage and responsible for the AP repolarization. Finally, we found no differences in the post-train AHP amplitudes, indicating unaltered contribution of the Ca^{2+}-dependent K^+ conductances activated during repetitive firing.

Functional implications of altered SCs and FCs firing during early symptomatic AD

We provide evidences for the presence of early, and therefore minimal differences in the firing properties of SCs and FCs of Tg2576 mice. This is crucial since it could impact information conveyed to the hippocampus via the perforant pathway, thus contributing to early cognitive AD deficits. At the age studied here, Tg2576 mice are specifically characterized by an over-production of different amyloid-β (Aβ) peptides, like $Aβ_{40}$ and $Aβ_{42}$, in the MTL [11, 18, 44]. It is thus likely that the reduced firing rates of FCs observed in Tg2576 mice derive from the accumulation of these peptides and their toxic effects on neuronal behaviour [31, 36]. Future experiments will clarify this issue.

SCs and FCs have been associated with the processing of spatial and non-spatial information, respectively, both of which significantly contribute to episodic memory formation [12, 47]. We thus suggest that spatial information conveyed by the SCs to the hippocampus is likely to remain relatively intact at this early stage of the pathology, while other sensory inputs, including olfactory information processing [30], conveyed by FCs to the hippocampus, might be altered. This conclusion is supported by observing that early olfactory dysfunction has been significantly associated with the risk of future AD and AD neuropathology burden in the brain of mice and patients [12, 24, 38, 52, 54]. Our data support the development of investigative methods aiming at detecting the early symptoms of AD on brain function by focusing on the memory processes that involve the LEC region and the subsequent processing of non-spatial stimuli. Finally, a recent work demonstrated that the FCs LEC to DG projecting neurons form a major input to the new born neurons of this hippocampal area [50]. Alterations of their firing patterns during early AD symptomatology could contribute to altered neurogenesis [49] and the development of cognitive deficits [9].

In conclusion, we defined the excitability profiles of SCs and FCs in adult mice showing that, as in adult rat, these two neuron populations display different firing properties. We also demonstrated that the excitability profile of LEC FCs of early symptomatic Tg2576 mice is more altered than MEC SCs, suggesting that LEC function might be first affected during AD progression.

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Fig. 1 - During sustained tonic firing, the AP firing frequency of SCs is higher than that of FCs, and AP shape differs significantly among the two neuronal cell types in WT mice.

A) Representative transmitted light microscopy image of a freshly prepared hippocampal-entorhinal cortex slice. Inserts represent magnified images of two SCs about to be patched, displaying characteristic stellate shape morphology typical of both SCs and FCs. **B, C**) Two representative traces of sustained tonic firing recorded from SCs (B, n=19) and FCs (C, n=17) are compared. No repetitive firing was recorded in the absence of injected current. **D**) Histogram distribution of interspike intervals (ISI) duration in SCs (black) and FCs (grey). **E, F**) While the average firing frequency was not significantly different (see text), the coefficient of variation (CV) and ISI values were significantly higher in SCs (**$P< 0.01$). **G**) AP parameters evaluated as summarized in Table 1. **H**) Two representative APs of SCs and FCs (taken from traces in B and C, and indicated by the asterisks) demonstrating that fAHP, DAP and overshoot values are significantly less pronounced in FCs compared to SCs. The dotted line corresponds to the 0mV value.

Table 1 – Summary of action potential (AP) parameters measured during tonic firing in SCs and FCs from WT and Tg2576 mice

Mean AP kinetic parameters calculated for SCs and FCs from adult WT (*****P< 0.05, WT-SCs vs WT- FCs) and Tg2576 mice (**#***P*<0.05, TG2576-SCs vs WT-SCs). Neurons were stimulated by injecting a minimum amount of current (200 pA) enough to induce membrane depolarization between -45 and -50 mV and obtain sustained tonic firing (SCs, n= 19; FCs, n= 17), as shown in Fig. 1B and C.

Fig. 2 - Firing frequency analysis and firing adaptation properties of SCs and FCs in adult WT mice.

AP firing frequency evaluated by injecting increasing steps of current (300-550 pA). **A**) Samples of AP trains in response to 300 and 550 pA current steps recorded from SCs (top) and FCs (bottom). It is evident that, while a 300 pA current step induces a firing of comparable frequency in SCs and FCs, a 550 pA current step reveals higher firing frequency in SCs compared to FCs. **B**) Mean firing frequency versus injected current for SCs (n=24) and FCs (n=23). At frequencies above 45 Hz, SCs fire significantly faster than FCs (**P*< 0.05). **B_1)** Rheobase values are significantly higher in SCs (**P*<0.05) **C**) The instantaneous firing frequency (f_o) measured between the first and second AP of the spike train plotted versus the injected current in SCs and FCs (**P*< 0.05; ***P*< 0.01). **D**) The instantaneous firing frequency (f_{ss}) measured between the last two APs of the spike train plotted versus the injected current in SCs and FCs.

Fig. 3 - Sodium channels availability is comparable in SCs and FCs.

A) Mean values of dV/dt_{max} calculated from the "phase plan plots" of panel **B** and **C** using the AP trains induced by 550 pA current steps for SCs and FCs. No significant differences were observed between the two groups of neurons. **B**) "Phase plane plots" obtained by plotting dV/dt vs voltage in SCs (top) and FCs (bottom) considering the first and the last APs of the train spike. **C**) Mean values of the spike threshold values taken as 4% of dV/dt_{max} in SCs and FCs. Threshold values of the first and last APs of the spike train were considered.

Fig. 4 - Time-dependent inward rectification potential, fAHP and post-train AHPs were higher in SCs with respect to FCs of adult WT mice, while no differences in AP half-width were observed.

A) Example trace of the time-dependent inward rectification potential measured in SCs and FCs during a 500pA hyperpolarizing current injection lasting 600ms. The recovery from hyperpolarization is higher in SCs probably due to an increased hyperpolarization-activated inward current (I_h). Notice that, in SCs, I_h is responsible for triggering spike firing at the end of the hyperpolarizing current step, when I_h channels deactivate. The point of the traces where peak, steady-state and τ_{on} values were measured are indicated by arrows. **A1**) The mean time constant (τ_{on}) of inward rectification potential activation is larger in FCs with respect to SCs (***P*< 0.01). **B**) Peak and steady-state values of membrane hyperpolarization (V_m-V_h where V_h=-60mV) plotted vs the negative current amplitude (from -100 to -1000 pA). While no differences were observed for peak values, the steady-state hyperpolarization in SCs (n= 22) were significantly lower than in FCs (n= 21) (**P*< 0.05; ***P*< 0.01). **C**) Example trace of AP trains generated by injecting 1nA for 200ms. The points of the traces where fAHP (fast afterhyperpolarization) and post-train AHP (afterhyperpolarization) values are measured are indicated by arrows. In the inset is shown the higher AHP, characteristics of SCs. **D, E**) The fAHP (**D**) and half-width (**E**) values (n=20 for SCs, n=19 for FCs; ***P*< 0.01, ****P*< 0.001) calculated for each AP of the spike train generated by current steps of 550 pA. **F**) Post-train AHPs plotted against the injected current (n= 20 for SCs, n= 19 for FCs; **P*< 0.05).

Fig. 5 - SCs of early symptomatic Tg2576 mice exhibit only a slight firing frequency alteration without changes of firing adaptation properties and sodium channels recruitment.

A, B) Two representative AP tonic firings recorded from WT (n= 19) and Tg2576-SCs (n= 18). **C**) Histogram distribution of the ISI duration of WT (black) and Tg2576-SCs (grey). While the average firing frequency was found not significantly different (see text), the ISI values (**E**) were significantly larger (***P< 0.001) in WT SCs thus unmasking the tendency of Tg2576-SCs to fire at a higher frequency. **F**) AP responses during injection of 550 pA current steps in WT (top) and Tg2576-SCs (bottom). **G**) Mean firing frequencies plotted versus injected current in WT (n= 24) and Tg2576-SCs (n= 24). **G_1**) Rheobase values are similar in both WT and Tg2576-SCs. **H, I**) f_o and f_{ss} values measured as in Fig. 2C and D were undistinguishable in SCs of WT and Tg2576 littermates. **L**) The dV/dt_{max} of the first and last AP of the spike trains (550 pA current step) measured as in Fig. 3 were nearly identical in SCs of WT (n= 22) and Tg2576 littermates (n= 22). **M**) The threshold of AP up-stroke (4% of dV/dt_{max}) of the first and the last AP were also similar in SCs of WT (n= 22) and Tg2576 littermates (n= 22).

Fig. 6 - The rectification potentials, within train fAHPs and post-train AHP of SCs of early symptomatic Tg2576 mice are comparable to those of WT littermates.

A) The time-dependent inward rectification potential, measured by plotting both the peak and steady-state values of membrane hyperpolarization (V_m-V_h where V_h=-60mV) vs the amount of injected current, is not different between the two genotypes (WT-SCs, n= 22; Tg2576-SCs, n= 22). **B, C**) fAHP and half-width values measured as in Fig. 4D and E (WT-SCs, n= 22; Tg2576-SCs, n= 21). **D**) post-train AHPs were plotted versus the amount of injected current as in Fig. 4F (WT-SCs, n= 22; Tg2576-SCs, n= 21).

Fig. 7 - FCs of early symptomatic Tg2576 mice fire at a lower rate and display increased firing adaptation with no differences in sodium channels recruitment

A, B) Representative traces of tonic firing recorded from FCs of WT (n=17) and Tg2576 (n=18) mice. **C**) Histogram distribution of the ISI duration in WT- (black) and Tg2576-FCs (grey). The average firing frequency was significantly lower in Tg2576-FCs (see text), as well as the ISI values (***P*<0.01) (**E**). The coefficient of variation (CV) of the two distributions (**D**) was not significantly different. **F**) Sample AP responses obtained during 550 pA current steps are displayed for WT-FCs (top) and Tg2576-FCs (bottom). **G**) Mean firing frequencies of FCs in WT (n=24) and Tg2576 littermates (n= 21). FCs of Tg2576 mice consistently fire at a lower frequency than FCs of WT littermates (**P*< 0.05). **G_1**) Rheobase values are significantly higher in Tg2576-FCs (***P*<0.01) **H**) The instantaneous firing frequency between the first two APs of the spike train (f_o), is similar in FCs of WT and Tg2576 littermates. **I**) The instantaneous firing frequency between the last two APs of the spike train (f_{ss}), is consistently lower in FCs of Tg2576 mice with respect to FCs of WT littermates (**P*< 0.05). **L**) The dV/dt_{max} calculated for the first and the last APs of the spike train (550pA) is comparable in FCs of WT (n= 22) and Tg2576 (n= 23) littermates. **M**) The threshold of AP upstroke (4% of dV/dT_{max}) of the first and the last APs of the spike train (550 pA) is comparable in FCs of WT (n= 22) and Tg2576 (n= 22) littermates.

Fig. 8 - FCs of early symptomatic Tg2576 mice display unaltered time-dependent inward rectification potential and post-train AHPs, but increased fAHP and decreased AP half-width during spike trains.

A) The time-dependent inward rectification potential, measured by plotting the peak and steady-state values of membrane hyperpolarization (V_m-V_h where V_h=-60mV) vs the amount of injected current was not different in the two genotypes (WT-FCs, n= 22; Tg2576-FCs, n= 18). **B**) Examples of AP trains generated by a 550 pA current step of 200ms. The within-train fAHP was measured as indicated by the arrow. The examples show that the fAHP in FCs of Tg2576 mice is higher than in FCs of WT littermates. **C, D**) Evolution of fAHP and half-width in WT-FCs (n= 24) and Tg2576-FCs (n= 21) within spike trains. From the third AP of the spike train, Tg2576-FCs displayed a significant increase in the fAHP (**P*< 0.05; ***P*< 0.01). For all APs of the spike train, Tg2576 FCs also displayed a significant decrease in the half width (**P*< 0.05; ***P*< 0.01). **E**) Mean post-train AHP values in Tg2576-FCs (n= 21) are comparable to those of WT-FCs (n= 24).