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Smaller quantal size and faster kinetics of single exocytotic events in chromaffin cells from the APP/PS1 mouse model of Alzheimer's disease

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ABSTRACT

The kinetics of single-amperometric exocytotic events has been measured in chromaffin cells of C57 mice and in an APP/PS1 mouse model of Alzheimer's disease (AD). K^+ depolarisation causes a burst of spikes that indicate the quantal release of the single-vesicle content of catecholamine. The kinetic analysis of 278 spikes from 10 control cells and 520 spikes from 18 APP/PS1 cells shows the following features of the latter compared with the former: (i) 45% lower $t_{1/2}$; (ii) 60% smaller quantal size; (iii) 50% lower decay time. Spike feet also showed 60% smaller quantal size. Immunofluorescence and thioflavin staining showed no amyloid beta (Aβ) burden in adrenal medulla slices of APP/PS1 mice that however exhibited dense Aβ plaques in the cortex and hippocampus. Furthermore, acetylcholinesterase staining of adrenal medulla indicated no apparent differences in the innervation by splanchnic cholinergic nerve terminals of chromaffin cells from control and APP/PS1 mice. This is the first report identifying subtle differences in the last steps of exocytosis that could be an indication of synaptic dysfunction of the secretory machinery not linked to Aβ burden in AD.

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1. Introduction

Pathogenic hallmarks of Alzheimer's disease (AD) are amyloid beta (Aβ) deposition to form senile plaques and hyperphosphorylation of tau protein with formation of neurofibrillary tangles. These abnormal processes cause impairment in neuronal and synaptic function probably resulting in deficits of neurotransmitter release and cognition [1,2]. Synaptic deficits may be due to presynaptic impairment of neurotransmitter synthesis, storage or release, and/or to a decrease of postsynaptic receptor density or modification of the coupling between receptors and intracellular signalling pathways. Both pre- and postsynaptic alterations may lead to synaptic loss, which is believed to be the major correlate of the severity of AD [3,4].

The multiprotein protease complex γ-secretase is involved in the regulation of many cellular processes. Thus γ-secretase, through its catalytic subunit presenilin (PS), cleaves Notch, N and E cadherins and accessory subunits of ion channels playing roles in development, cell adhesion and ion channel modulation respectively [3]. Moreover PS1, independently of its catalytic activity, has been suggested to participate in ER Ca^{2+} homeostasis [5]. Interestingly, PSs also seem to play a critical role in regulating neurotransmitter release [6]. γ-Secretase also intervenes in the processing of amyloid precursor protein (APP) and production of Aβ peptide in non-pathological cell metabolism [7]. Although its physiological function is not clear, it is believed that Aβ is involved in the regulation of normal synaptic function as well as in the chelation of metal ions in the brain [8,9]. Therefore alterations in these proteins may produce per se synaptic changes that may underlie AD pathogenesis, as well as the induction of amyloid plaque deposition.

Dozens of mutations in PS-1 have been associated with early onset familiar Alzheimer disease (FAD). These mutations may act in a combined manner contributing to the development of AD: By disrupting Ca^{2+} handling and regulation of neurotransmitter release they may produce synaptic dysfunction and loss; additionally, mutated PS-1 increases the production of $A\beta_{1-42}$ leading to formation of toxic amyloid plaques characteristic of the disease, which further affect synaptic transmission and cause neurodegeneration. Mutations in APP, which produce an increase in the ratio of $A\beta_{1-42}/A\beta_{1-40}$ are also associated with FAD.

In this study we have used aged mice which coexpressed two FAD-linked mutations, the human PS1 variant (A246E) and the so called Swedish mutation of APP [10]. This mouse model shows an accelerated Aβ deposition [10], impairment of synaptic

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plasticity [11], accelerated decay of LTP [12] and of excitatory synaptic transmission in the hippocampus [13].

Chromaffin cells (CCs) of the adrenal medulla are modified sympathetic neurons that release catecholamines upon splanchnic nerve stimulation [14] as a response to acute stress. There is a complete absence of data on possible peripheral alterations that may provide early warnings of the initiation of the disease. Furthermore, proteins key in the disease, such as PS1, APP and Aβ are expressed in CCs [15–18]. Thus, APP and Aβ have been reported to be stored in chromaffin granules and to be released upon depolarizing stimuli with cholinergic agonists or high K^+ [19]. In addition AD progression has been linked to acute stress [20].

The exocytotic machinery is a complex proteic structure that is similar in neurons and many neurosecretory cells. Obviously, the search for synaptic dysfunctions in AD has been focused on the brain [21]. However, the analysis of the last steps of exocytosis in the millisecond-time range has been best studied in peripheral chromaffin cells, using amperometry [22]. This technique provides information on subtle differences in fast exocytotic mechanisms, which have not been possible to study in CNS. Here we have compared the kinetic parameters of single-vesicle amperometric events in chromaffin cells from control C57 mice and the APP/PS1 transgenic mouse model of AD. Given that cholinergic neuron loss is prominent in AD brains, we used the AChE activity technique to assess alterations in both, the cholinergic innervation and the activity of the enzyme itself which is substantially depleted in the brains of patients with AD [23]. Finally we assayed the presence of amyloid plaque deposition in the adrenal medullae and brain of these mice.

2. Materials and methods

2.1. Animals

Double mutants for APP and PS1 (APPswe/PS1-A246E) and the age-matched wild-type C57 controls were a generous gift from Dr. Ricardo Martínez-Murillo (Instituto Cajal, CSIC, Madrid, Spain). They were obtained as described [24]. Eight adult male and female mice aged 20 months were used. The experimental procedures adhered to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Ethical Committee for the Care and Use of Animals in Research of the Universidad Autónoma de Madrid, in accordance with the European Communities Council Directive 86/609/EEC and with the Spanish Royal Decree RD 1201/2005.

2.2. Chromaffin cell culture and amperometric recordings

Chromaffin cells were prepared as previously described [25]. Experiments were carried out in cells 1–3 days old. Amperometry recordings were performed by placing a carbon fibre electrode held at +730 mV by means of an EPC-10 amplifier (HEKA, lambrecht, Germany), in the vicinity of the cell.

2.3. Histology

For the histological assessment, mice were anesthetized with 3% isoflurane in oxygen under spontaneous respiration, transcardially perfused with saline solution and then fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains and adrenal glands were post-fixed in the same fixative, cryoprotected with 30% sucrose in 0.1 M phosphate buffer, pH 7.4, and embedded in OCT™ (Sakura Finetek, Netherlands) before sectioning on a cryostat (Leica CM 1950, Wetzlar, Germany) in 20-μm coronal brain sections that included the dorsal hippocampus, and transversal adrenal sections that included the adrenal medulla. Sections were stained for cholinesterase to study adrenal medulla cholinergic innervation. For Aβ staining, sections were washed and then immersed in 0.1% thioflavin S in 50% ethanol, followed by 80% ethanol, washed in distilled water and mounted in aqueous medium. For immunofluorescence, sections were washed in PBS, blocked with 10% normal goat serum and incubated overnight with the primary antibody mouse anti-human Aβ (1:100, AbD Serotec, Kidlington, UK). Sections were then incubated with fluorochrome-conjugated secondary antibody (Alexa 647, Invitrogen, Carlsbad, CA) counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA) and mounted in aqueous medium. Negative control sections were incubated without the primary antibody. Sections were mounted and analysed under bright field light or fluorescence in a microscope (Leica DMI6000 B, Wetzlar, Germany).

2.4. Data analysis and statistics

Analysis of single amperometric spike kinetics was carried out as previously described [25] using an automated macro [26] written in Igor (Wavemetrics, Oregon, USA). Median and mean values for all the spikes of each cell were obtained and then pooled together for statistical comparison; this method helps overcoming the large variability is spike number and spike kinetics by giving each cell the same weight independently of the number of spikes produced. Appropriate statistical tests were used.

3. Results and discussion

Chromaffin cells secrete catecholamines by a calcium-dependent exocytotic mechanism. Calcium entry though voltage-dependent calcium channels that open during cell depolarisation is the main mechanism that couples the stimulus to the secretory response. We used here 70 mM K^+ (low Na^+) to stimulate the quantal release of catecholamine from control and APP/PS1 chromaffin cells; this K^+ concentration drives the resting membrane potential from around −60 mV to 0 mV [27], causing a sharp increase of Ca^{2+} entry and the rapid exocytotic release of catecholamine.

When stimulated with a 10-s 70 K^+ pulse, a burst of secretory spikes were produced in both control and APP/PS1 chromaffin cell (Fig. 1A and B). We analysed the events generated by the K^+ pulse only in those cells that produced 20 or more resolvable single spikes larger than 5 pA. Thus, for the analysis we selected 278 spikes from 10 control cells and 520 spikes from 18 APP/PS1 cells. Hence, the number of spikes per cell and per stimulus was similar in control (28.4 ± 3, mean ± SE) and APP/PS1 (34.1 ± 4.5) cells.

Fig. 1C shows a scheme of a prototype amperometric spike with the different kinetic parameters analysed. The slow foot preceding the fast spike is an indication of the initial phase of fusion pore opening and indirectly, of the initial steps of vesicle fusion [28]. Table 1 summarises the kinetic parameters of the foot that was found in about 60% of the spikes analysed of both cell types. The foot amplitude (I_{foot}) was 30% smaller in APP/PS1 compared with control cells; however, this difference did not reach the level of statistical significance. A similar diminution (about 35%) was observed in foot duration (T_{foot}) but again this was at the limit of statistical significance. Taken together, the decrease of I_{foot} and T_{foot} suggested a smaller foot charge (Q_{foot}). Indeed in control cells Q_{foot} was 60 fC versus 28 fC in the transgenic ($P < 0.01$). In other words, the quantity of catecholamines that were released during the initiation of the fusion pore was 50% smaller in APP/PS1, compared with control C57 mouse chromaffin cells.

Expansion of the fusion pore gives rise to the fast amperometric spikes, that were analysed as shown in Fig. 1C and are summarised in Table 2. The rise rate was around 140 pA/ms for both control and APP/PS1 cells. Of note is the fact that this rate of catecholamine re-

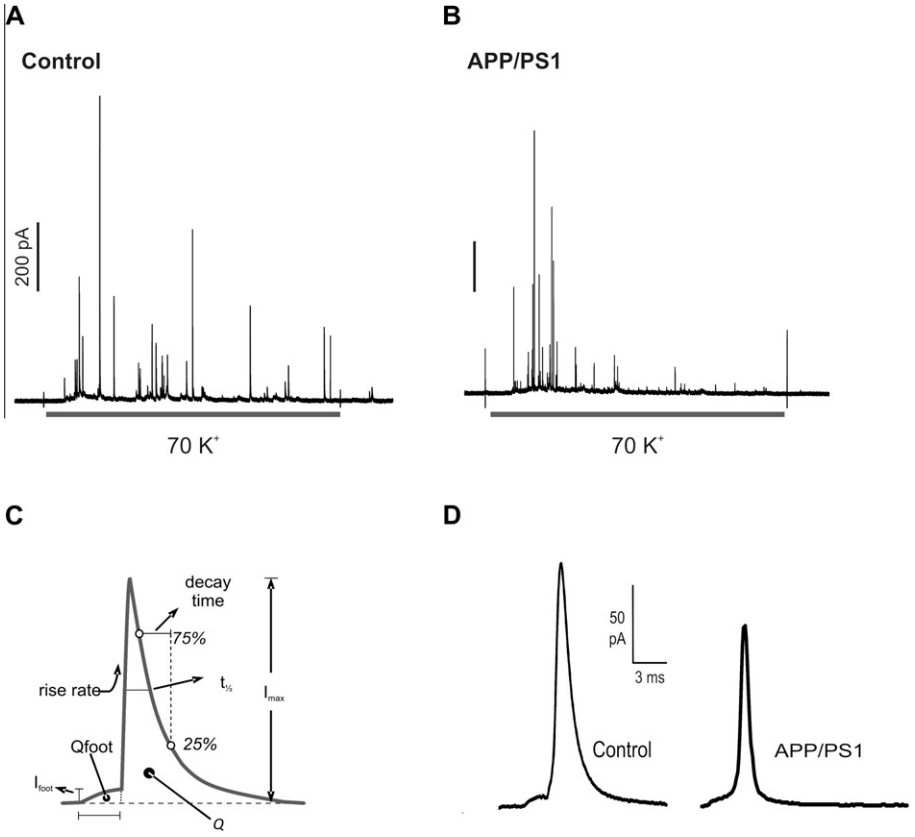

Fig. 1. Exocytotic response to high K^+ depolarization measured with amperometry. A 10 s 70 mM K^+/low Na^+ was applied to control (panel A) and APP/PS1 (panel B) chromaffin cells. C, Depiction of the spike and foot kinetic parameters analysed. (Panel D) Average control and APP/PS1 spikes.

Table 1
Kinetic parameters of feet found at the beginning of single amperometric events in the burst spike traces elicited by 70 K^+ stimulation of C57 control and APP/PS1 mouse chromaffin cells.

Cell type	% Spikes with foot	I_{foot} (pA)	T_{foot} (ms)	Q_{foot} (fC)
Control	63 ± 5.22 (66.22)	7.33 ± 1.25 (6.63)	5.82 ± 0.75 (5.32)	59.5 ± 9.16 (60.3)
APP/PS1	62.49 ± 3.8 (60.98)	5.28 ± 0.6 (5.41)	4.09 ± 0.34 (3.59)	27.83 ± 4.35** (23.06)

Control and APP/PS1 (rows) were compared for each single spike parameter (columns) using the non-parametric Mann–Whitney rank sum test. Significant differences between both cells types (APP/PS1 row): $^{**}P < 0.01$. Data are presented as means ± SE; median is also shown, below mean, between parentheses. Statistical tests were performed using the mean of at least 20 spikes per cell. $^{**}P$-value <0.01.

Table 2
Single-spike kinetic parameters calculated from the 70 K^+ elicited quantal catecholamine release responses in chromaffin cells from control and APP/PS1 mice.

Cell type	Events (cells)	Rise (pA/ms)	I_{max} (pA)	$t_{1/2}$ (ms)	Q (pC)	Decay time 75–25% (ms)
Control	278 (10)	144 ± 36.8 (112)	141 ± 24.3 (125)	6.29 ± 0.62 (8.89)	1.00 ± 0.09 (1.01)	7.02 ± 0.8 (6.73)
APP/PS1	520 (18)	135 ± 19.5 (118)	106 ± 12.4 (95)	3.73 ± 0.43** (3.27)	0.45 ± 0.05*** (0.48)	4.04 ± 0.54** (3.06)

Control and APP/PS1 chromaffin cells (rows) were compared for each single spike parameter (columns) using the nonparametric Mann–Whitney rank sum test. Significant differences between both cell types are symbolised with asterisks in the APP/PS1 row. Data are presented as means ± SE; median is also shown, below mean, in parentheses. Statistical tests were performed using the mean of at least 20 spikes per cell for each parameter. $^{**}P$-value <0.01, $^{***}P$-value <0.001.

lease is near 3-fold higher that stimulated for the quantal release of catecholamine in the rat chromaffin cell stimulated also with 70 mM K^+ [25]. With regard to the spike amplitude (I_{max}), although in APP/PS1 cells I_{max} was 30% lower (106 pA) than in control cells (141 pA), this difference was not statistically significant.

More significant were the differences in spike half-width ($t_{1/2}$), quantal size (Q) and decay time. Thus, $t_{1/2}$ in APP/PS1 was 3.7 ms, about 45% shorter than in control cells (6.3 ms, $P < 0.01$). Total charge (Q), an indication of catecholamine vesicle content, was 0.45 pC in APP/PS1 and 1 pC in control spikes, indicating that the quantal size of APP/PS1 spikes was about half that of control spikes. This is coherent with values of I_{max} and $t_{1/2}$. The spike decay time 75–25% amounted to 4 ms in APP/PS1 and 7 ms in controls.

Fig. 1D presents an average shape of the control and APP/PS1 spikes analysed. The APP/PS1 amperometric spike is smaller and thinner, a feature possibly linked to the substantial reduction of the quantal catecholamine content of the dense-cored vesicles of chromaffin cells from mutated mice, compared with control C57 mice.

This alteration of the exocytotic event could be linked to differences in the innervation of chromaffin cells or to Aβ burden of the

adrenal medulla of APP/PS1 mice. The acetylcholinesterase (AChE) enzymatic technique revealed the typical dense adrenal medullary innervation of chromaffin cells by cholinergic nerve endings of incoming splanchnic fibres [14]. No apparent differences in innervation were seen between APP/PS1 slices (Fig. 2A) and control slices (Fig. 2B). Concerning Aβ burden, this peptide was not detected in adrenal medullae from APP/PS1 mice neither with thioflavin staining (Fig. 2C) nor with anti-Aβ antibodies (Fig. 2D). In contrast, in the hippocampus of the same animals, large and abundant Aβ plaques were revealed with both thioflavin staining (Fig. 2E) and with fluorescent anti-Aβ antibodies (Fig. 2F). Thus, it seems that the quantal exocytotic events that had a smaller quantal size and shorter duration in APP/PS1 mice are not linked to the Aβ pathology typical of this AD mouse model namely, Aβ deposition and synaptic deficits.

Vesicle fusion during exocytosis in chromaffin cells is triggered by overlapping actions of two Ca^{2+} sensor proteins, synaptotagmin-1 and -7 (Syt1 and Syt7) [29]. Interestingly a point mutation in the C2B domain of Syt7 reduces patch-amperometry recorded spike's $t_{1/2}$ and Q by 50% [30]. This is very similar to the difference between APP/PS1 and control mice we describe here. Whether this alteration is linked to a functional deficit or a pathophysiological modulation of the C2B domain of Syt7 is an interesting possibility that deserves attention in the search of new therapeutic targets in AD.

Adrenaline (AD) and noradrenaline (NA) are profusely released from chromaffin cells during acute stress. In this respect Aβ formation has been linked to acute stress in a stress model in rats [20]. β2 agonists increased stress-induced Aβ burden whilst β2 antagonists slowed Aβ production. Yu et al. [20] proposed β2 receptors as a possible target for the restraining of AD progression. In this study we show that, in the APP/PS1 model of AD, the mean quantal content released per vesicle is halved. It is tempting to suggest that the physiological meaning of this reduced release of catecholamines is a protective reaction in an attempt to decrease the toxic and rapid accumulation of Aβ in the brains of the transgenic animals.

In summary, we describe here alterations in the last steps of regulated exocytosis in a peripheral neuroendocrine cell of a mouse model of AD. Due to accessibility and available methodological approaches to study minute changes of the kinetics of the last steps of exocytosis, chromaffin cells may become invaluable models to study the potential changes of exocytotic machine in the var-

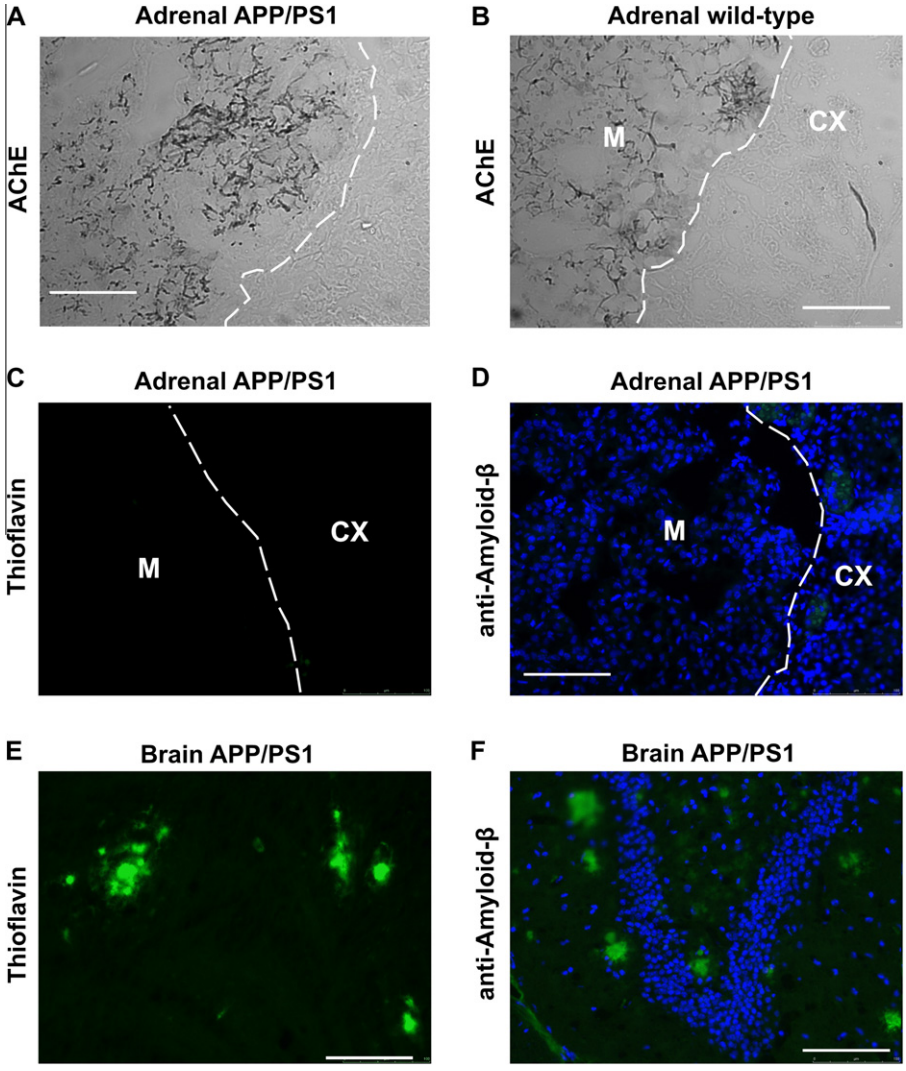

Fig. 2. Aβ load in adrenal medullae and hippocampus of control and APP/PS1 mice. Top, Acetylcholinesterase-stained adrenal glands of APP/PS1 (panel A) and wild-type mice (panel B). Panels C and E, adrenal and hippocampal sections from APP/PS1 mice, stained either with thioflavin S (C and E, green) or labelled with anti-Aβ antibody (D and F, green) and counterstaining nuclei with Hoechst (blue). M, adrenal medulla; CX adrenal cortex. Scale bars 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ious mouse models of AD available. These studies would aid to shed light on other central pathophysiological alterations produced by the disease.

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