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Depressed excitability and ion currents linked to slow exocytotic fusion pore in chromaffin cells of the SOD1\textsuperscript{G93A} mouse model of amyotrophic lateral sclerosis

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Depressed excitability and ion currents linked to slow exocytotic fusion pore in chromaffin cells of the SOD1\textsuperscript{G93A} mouse model of amyotrophic lateral sclerosis. The excitotoxic hypothesis implies that a chronically hyperexcitable motoneuron would fire more action potentials (APs), and consequently, more \(\text{Ca}^{2+}\) will enter into its cytoplasm, eventually eliciting its death (33, 39, 70). Some reports suggest that embryo motoneurons and cortical neurons of mSOD1 mice are hyperexcitable (39, 55, 57). However, other reports show normal excitability at neonatal age (58) or even hypoexcitability at adult P30-P80 mSOD1 mice (15).

The selective loss of motoneurons is central stage in the pathogenesis of amyotrophic lateral sclerosis (ALS). This leads to muscle weakness, atrophy, and spasticity that end up into paralysis and respiratory insufficiency; the disease is progressive and the mean duration of survival is 3 to 5 yr (60). Most ALS cases are sporadic, but ~10% of patients have a positive family history; of these, 20% have mutations in the gene for \(\text{Cu}^{2+}/\text{Zn}^{2+}\) superoxide dismutase 1 (SOD1). When modeled in mice, the mutation G93A (glycine to alanine at codon 93; mSOD1) shows adult disease onset from postnatal day 90 (P90) onwards and reproduces the clinical paralytic symptoms of ALS (25, 29), with significant loss of motoneurons (10). Why motoneurons die in ALS is unknown. The excitotoxic hypothesis implies excess glutamate release in both patients and transgenic mice (6, 37, 59, 64, 68). This will lead to altered \(\text{Ca}^{2+}\) homeostasis, excess production of free radicals, and apoptotic death of motoneurons (21, 51, 70). Consistent with this is the fact that riluzole, the only medicine so far approved to treat ALS (2, 60), inhibits the exocytotic release of glutamate (44). Also, it has been reported that cerebrospinal fluid of ALS patients augments the basal concentration of cytosolic calcium ([Ca\textsuperscript{2+}]),\textsubscript{c} and elicits motoneuron death (74).

The excitotoxic hypothesis implies that a chronically hyperexcitable motoneuron would fire more action potentials (APs), and consequently, more \(\text{Ca}^{2+}\) will enter into its cytoplasm, eventually eliciting its death (33, 39, 70). Some reports suggest that embryo motoneurons and cortical neurons of mSOD1 mice are hyperexcitable (39, 55, 57). However, other reports show normal excitability at neonatal age (58) or even hypoexcitability at adult P30-P80 mSOD1 mice (15).

We here propose the hypothesis that a more general alteration in the fine tuning of neuronal excitability and the exocytotic machinery may underlie the pathogenic features of ALS and that these alterations could also be present in various neuronal types and neuroendocrine cells including adrenal chromaffin cells. In fact, a few early reports have found the presence of sympathetic hyperactivity in ALS patients (9, 73) although others found no changes (61). Increased levels of norepinephrine in blood and cerebrospinal fluid were also found in ALS patients (75). Additionally, later studies found that ALS patients were hypermetabolic, a state involving mitochondrial energy production or even sympathoadrenal activation (16). Recently, a report revealed a differential steroid profile with age and gender in ALS patients.
relative to controls (27). Lastly, a study in the ALS mouse model carrying the mutation G86R of SOD1 has found early progressive disturbances in the neurohypophysial axonal transport of neurosecretory products from neuronal perikarya to nerve terminals (28).

All these alterations in neurosecretory systems could have in common a dysfunction of the exocytotic machinery and the last steps of exocytosis. Sympathetic neuronal-like chromaffin cells are increasingly being used to explore these questions. For instance, altered exocytosis in chromaffin cells has been shown in the APP/PS1 mouse model of Alzheimer’s disease (AD) (14); in the knockout mouse model of the huntingtin-associated protein 1 (HAP1), a model of Huntington’s disease (HD) (41); and in mice carrying specific mutations in proteins of the exocytotic machine (HAP1) to model carrying the mutation G86R of SOD1 has found early progressive disturbances in the neurohypophysial axonal transport of neurosecretory products from neuronal perikarya to nerve terminals (28).

C2 ALTERED FUSION PORE KINETICS IN THE SOD1G93A MICE

MATERIALS AND METHODS

Animals. Experiments were conducted according to the recommendation of the Ethics Committee from Universidad Autónoma de Madrid on the use of animals for laboratory experimentation in accordance with the code of ethics and guidelines established by European Community Directive (2010/63/EU) and Spanish legislation (RD53/2013). All efforts were made to avoid animal suffering and to use the minimum number of animals allowed by the experimental protocol and the statistical power of group data. Mice were housed individually under controlled temperature and lighting conditions with food and water provided ad libitum and housed on a 12:12-h light cycle.

Male B6.Cg-Tg(SOD1-G93A)1Gur/J mice, hereafter referred to as mSOD1, were purchased from The Jackson Laboratory, Bar Harbor, ME (stock no. 004435). These mice overexpress a point mutated form mSOD1, were purchased from The Jackson Laboratory, Bar Harbor, ME (stock no. 004435). These mice overexpress a point mutated form

penicillin, and 50 µg/ml streptomycin were added to each well and remained in the incubator for 1–2 days during which experiments were done.

Monitoring of cell excitability and ion currents. Recordings of membrane potential (Vm) and APs were made under the current-clamp mode in the whole cell configuration of the patch-clamp technique (30), which allows the observation of spontaneous variations in the Vm. Cells were superfused with control Tyrode solution at pH 7.4 containing the following (in mM): 137 NaCl, 1 MgCl2, 2 CaCl2, and 10 HEPES/NaOH; an intracellular solution at pH 7.4 containing the following (in mM): 135 KCl, 10 NaCl, 10 HEPES, 1 MgCl2, and 5 MgATP was introduced in the patch-clamp pipette.

Inward currents through nicotinic receptor ion channels (INa), voltage-activated sodium channels (INa), voltage-activated calcium channels (ICa), voltage-activated potassium channels [IK(Ca)], and calcium-dependent potassium channels [IK(Ca)] were recorded using the voltage-clamp mode of the whole cell configuration of the patch-clamp technique (30). Whole cell recordings were made with fire-polished borosilicate pipettes (resistance 2–5 MΩ) that were mounted on the headstage of an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany), allowing cancellation of capacitative transients and compensation of series resistance. Data were acquired with a sample frequency of 20 kHz by using PULSE 8.74 software (HEKA Elektronik). The data analysis was performed with Igor Pro (Wavemetrics, Lake Oswego, OR) and PULSE programs (HEKA Elektronik). Covariates containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. Cells were continuously superfused with a control Tyrode solution at pH 7.4 containing the following (in mM): 137 NaCl, 1 MgCl2, 2 CaCl2, and 10 HEPES/NaOH. Once the patch membrane was ruptured and the whole cell configuration of the patch-clamp technique had been established, the cell was locally, rapidly, and constantly superfused with an extracellular solution of similar composition to the chamber solution but containing nominally 0 mM Ca2+ to measure INa and 2 mM Ca2+ to measure INa, INa, and IK.

Cells were internally dialyzed with an intracellular solution containing the following (in mM): 100 CsCl, 14 EGTA, 20 TEA-Cl, 10 NaCl, 5 Mg-ATP, 0.3 Na-GTP, and 20 HEPES/CsOH (pH 7.3) for the recording of IINa, INa, and IINa, for recording IK the intracellular solution had the following composition (in mM): 135 KCl, 14 EGTA, 10 NaCl, 5 Mg-ATP, 0.3 Na-GTP, and 20 HEPES/KOH (pH 7.3). The external solutions were rapidly exchanged using electronically driven miniature solenoid valves coupled to a multibarrel concentration-clamp device, the common outlet of which was placed within 100 µm of the cell to be patched. The flow rate was 1 ml/min and was regulated by gravity.

For measuring the different currents, cells were held at −80 mV; INa was generated by 10-ms depolarizing pulses to −10 mV, INa was generated by 50-ms depolarizing pulses to −10 or 0 mV; IINa was generated by the application of 250-ms acetylcholine (ACh) pulses (100 µM); and IK was generated by the application of a 10-ms depolarizing command to +30 mV followed by a 400-ms full depolarization pulse to +120 mV. All experiments were performed at room temperature (24 ± 2°C) on cells from 1 to 2 days after culture.

Measurements of changes in the cytosolic calcium concentration. Chromaffin cells were incubated for 1 h at 37°C in DMEM medium containing the calcium probe fura-2 acetoxymethyl ester (fura-2 AM; 10 µM). After this incubation period, the coverslips were mounted in a chamber and cells were washed and covered with Tyrode solution. The setup for fluorescence recordings was composed of a Leica DMI 4000 B inverted light microscope (Leica Microsystems; Barcelona, Spain) equipped with an oil immersion objective (Leica 40× Plan Apo; numerical aperture 1.25). Once the cells were placed on the microscope, they were continuously superfused by means of a five-way superfusion system at 1 ml/min with a common outlet 0.28-mm tube driven by electrically controlled valves with Tyrode solution. Fura-2 AM was excited alternatively at 340 ± 10 and 387 ± 10 nm

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using a Küber CODIX xenon arc lamp (Leica). Emitted fluorescence was collected through a 540 ± 20 nm emission filter and measured with an intensified charge coupled device camera (Hamamatsu camera controller C10600 orca R2). Fluorescence images were generated at 1-s intervals. All experiments were performed at room temperature (24 ± 2°C) on cells from 1 to 2 days after culture.

Amperometric recordings. Quantal release of catecholamine was measured with amperometry (11, 72). Electrodes were built as previously described previously (36) by introducing a 10-μm diameter graphite fiber (Amoco, now part of BP-Group, London, UK) into glass capillary tubes (Kimble-Kontes, Vineland, NJ). These tubes were then pulled (Narishige PC-10 pipette puller; Narishige, Tokyo, Japan), and the carbon fiber was inserted in both thin ends of the pulled tube and was cut with a pair of small scissors obtaining thus two pipettes with a carbon fiber piece sticking out of each tip. The tip was sealed by a two-component epoxy (EPIKOTE 828-Miller-Stephenson, Danbury, CT) and m-phenylenediamine, 14% (Aldrich, Steinheim, Germany). The electrodes were left overnight to dry, introduced into an oven at 100°C for 2 h, and then kept another 2 h at 150°C. The amperometer was homemade (Segainvex UAM, Madrid, Spain) and connected to an interface (PowerLab/4SP; ADInstruments, Oxford, UK) that digitized the signal at 10 kHz sending it to a personal computer that displayed it within the Pulse v8.74 software (HEKA Elektronik). A 700-mV potential was applied to the electrode with respect to an AgCl ground electrode. The electrodes were calibrated following good amperometric practices (42) by perfusing 50 μM norepinephrine dissolved in standard Tyrode and measuring the current elicited; only electrodes that yielded a current between 200 and 400 pA were used. The coverslips were mounted in a chamber on a Nikon Diaphot inverted microscope used to localize the target cell, which was continuously superfused by means of a five-way superfusion system with a common outlet driven by electrically controlled valves, with a Tyrode solution composed of the following (in mM) 137 NaCl, 1 MgCl2, 5 KCl, 2 CaCl2, 10 HEPES, and 10 glucose (pH 7.4, NaOH). The high K+ solutions were prepared by replacing equiosmolar concentrations of NaCl with KCl. At the time of experiment performance, proper amounts of drug stock solutions were freshly dissolved into the Tyrode solution. All experiments were performed at room temperature (24 ± 2°C) on cells from 1 to 2 days after culture.

Immunoblotting and image analysis of the expression of proteins of the exocytotic machinery and the α3-subunit of nicotinic receptors. Motor cortex, hippocampus, spinal cord, and adrenal glands were removed from the mice, and membrane proteins were extracted using the MEM-PER Membrane Protein Extraction Kit (Thermo Scientific, Rockford, IL) following the manufacturer’s instructions. At all times, proteins were in the presence of protease inhibitors (Halt Protease Inhibitor Cocktail; Thermo Scientific). Proteins were quantified using the Bicinchoninic Acid Protein Assay (G-Biosciences, St. Louis, MO). Proteins (20 μg) were resolved by SDS-PAGE (12%) and transferred to Immobilon-P Transfer Membrane (Millipore, Billerica, MA). Membranes were blocked in Tris-buffered saline with 0.05% Tween-20 containing 4% bovine serum albumin and incubated for 2 h at room temperature with primary antibodies anti-synaptotagmin 1 (SYT1, 1:200), anti-synaptotagmin 7 (SYT7, 1:200), anti-synaptotagmin 1 (SYT1, 1:200), anti-synaptosomal-associated protein 25 (SNP25, 1:200), anti-vesicle-associated membrane protein 2 (VAMP2, 1:200), and anti-neuronal ACh receptor subunit α3 (NACHRA3, 1:200), all of them from Santa Cruz Biotechnology (Dallas, TX) and with anti-β-actin (1:100,000; Sigma-Aldrich, St. Louis, MO) as loading control; and then, for 45 min with secondary antibodies conjugated with peroxidase (1:10,000; Santa Cruz Biotechnology). The membrane was developed using the ECL Select Western Blotting Detection Reagent (GE Healthcare, Chalfont St. Giles, UK). Different band intensities corresponding to immunoblot detection of protein samples were quantified using Scion Image Alpha 4.0.3.2 program (Scion).

Rota-Rod test. The Rota-Rod apparatus (Stoelting, Wood Dale, IL) was used to assess motor performance. Mice were challenged with an initial speed of 8 rpm and an increase of 1 rpm each 8 s until they fell down. The time spent walking on the Rota-Rod was measured, and time from 10 repetitions was averaged; 180 s was chosen as the arbitrary cut-off time. Mice were trained for 1 day [training day (Trd)] to get acquainted with the Rota-Rod apparatus for the test day (Td) in consecutive days. Different mice at P90 and P130 ages were used.

Statistical analysis. Ion currents and cell excitability data analysis were performed using the GraphPad Prism version 5.01 for Windows (GraphPad Prism Software, San Diego, CA). Student’s t-test or one-way ANOVA followed by Tukey’s or Dunnett’s post hoc tests were used to determine statistical significance between means.

Fig. 1. Drastic reduction in the number of spontaneous action potentials in mSOD1 chromaffin cells compared with wild-type (WT) cells. A: example voltage record obtained from a WT cell. B: voltage record obtained from an example mSOD1 cell. C: average action potential number recount from 300-s records of WT and mSOD1 cells. D: average resting membrane potentials from WT and mSOD1 cells. Data in C and D are means ± SE of the number of cells and cultures shown in parentheses. ***P < 0.001, with respect to WT cells (unpaired Student’s t-test).
**P < 0.05 was taken as the limit of significance, and **P < 0.01 and ***P < 0.001 were taken as statistical significance.

Data from measurement of changes in the [Ca\(^{2+}\)] were obtained from LAS AF software and Ascent software version 2.4.2. Graphs and the mathematical analyses were performed using the GraphPad Prism software, version 5.01 (GraphPad Software). Areas were calculated by integrating the [Ca\(^{2+}\)] transient over time during the stimulus duration by means of Origin Pro 8 SR2 software, version 8.0891 (OriginLab, Northampton, MA). Areas were worked out by the integration of the input data set by using the trapezoidal rule. Results shown in the text and figures are expressed as means ± SE. Statistical analyses were carried out with one-way ANOVA test and Tukey post hoc analyses. *P < 0.05 was taken as the limit of significance, and **P < 0.01 and ***P < 0.001 were taken as statistical significance.

Amperometry data analysis was carried out on a personal computer using Excel software (Microsoft, Redmond, WA) and IgorPro software (Wavemetrics). Total amperometric quantal charge was calculated by integrating the amperometric current over time during the stimulus duration with a homemade macro written in IgorPro software. The number of spikes >5 pA was manually counted on an extended graph displayed in the computer screen. A ruler was drawn at 5 pA, and only the spikes going above the threshold amplitude were considered. The kinetic analysis of single amperometric events (spikes) was performed as previously described (24) using a macro written in IgorPro software (48). Median values for all the spikes of each cell were obtained and then pooled together for statistical comparison; this method helps overcoming the large variability in spike number and spike kinetics by giving each cell the same weight independently of the number of spikes produced. Differences between means of group data fitting a normal distribution were assessed by using either ANOVA or Kruskal-Wallis test for comparison among multiple groups or Student’s t-test for comparison between two groups. *P < 0.05 was taken as the limit of significance, and **P < 0.01 and ***P < 0.001 were taken as statistical significance.

Comparisons between groups from immunoblotting and image analysis of the expression of proteins, were performed by one-way ANOVA followed by the Newman-Keuls post hoc test or by the unpaired Student’s t-test using the Graph Pad Prism software version 5.01. **P < 0.05 was taken as the limit of significance, and ***P < 0.01 and ***P < 0.001 were taken as statistical significance.

Data from Rota-Rod test are means from 10 repetitions of Trd and Td of different mice at P90 and P130 ages. *P < 0.05 was taken as the limit of significance, and **P < 0.01 and ***P < 0.001 were taken as statistical significance.

Materials and chemicals. The calcium binding probe fura-2 AM was purchased from Invitrogen (Eugene, OR). ACh chloride and all other chemical components used in this study for cell cultures and the various experiments were obtained from Sigma-Aldrich and GIBCO-Invitrogen (Barcelona, Spain).

RESULTS

To discern about possible differences in the stimulus-secretion coupling process between WT and mSOD1 chromaffin cells, we explored the various steps in the chain of events involved in such a process. Those steps include chromaffin cell excit-
ability and the firing of spontaneous APs, ion currents, \([Ca^{2+}]_c\) transients, and the exocytotic release of catecholamine, including the kinetic analysis of the single-vesicle amperometric spike. With the thorough analysis of those events we expected to raise a hypothesis to explain the changes in the quantal release of catecholamines. Furthermore, although ACh and high K\(^+\) quantitatively elicit similar Ca\(^{2+}\)-dependent catecholamine release responses (19), we later on showed that the Ca\(^{2+}\) transients and the secretory responses elicited by ACh and K\(^+\) considerably differed (8). Thus we analyzed here the \([Ca^{2+}]_c\) transients and the kinetics of the quantal release of catecholamine in chromaffin cells from WT and mSOD1 mice stimulated with ACh or high K\(^+\).

**Excitability of chromaffin cells from WT and mSOD1 mice.** To determine the resting \(V_m\), cells were sealed under the voltage-clamp mode and held at a potential of \(-80\) mV until series resistance was <20 MΩ. Then, the amplifier was switched to current-clamp mode and current injection was set to 0 pA. In 15 cells from 6 different WT mice, \(V_m\) was \(-67.8 \pm 2.05\) mV, and in 31 cells from 7 different mSOD1, \(V_m\) was \(-62.85 \pm 1.9\) mV (Fig. 1D). These values agree with those found in chromaffin cells from various animal species that ranged between \(-50\) and \(-80\) mV (reviewed in Ref. 13).

In spite of the fact they had similar \(V_m\), the firing of spontaneous APs considerably differed in chromaffin cells of WT and mSOD1 mice. For instance, the example WT cell of Fig. 1A had a \(V_m\) of around \(-60\) mV and initially fired a burst of high-rate APs during the first 70 s; thereafter, the cell had silent periods with a few scattered APs. Figure 1B corresponds to an example mSOD1 cell with only a few APs scattered along the 5-min recording period. In 15 WT cells from 6 different mice, we counted 57.6 \pm 12 APs during the 5-min recording period; in sharp contrast, in the 31 mSOD1 cells from 7 mice we only counted 2.6 \pm 1 APs (Fig. 1C).

**Whole cell ion currents of chromaffin cells from WT and mSOD1 mice.** Whole cell ion currents were recorded in WT and mSOD1 chromaffin cells voltage clamped at \(-80\) mV. \(I_C\) were elicited by the application of a 250-ms pulse of an extracellular solution containing 100 µM ACh. ACh pulses were applied at regular 2-min intervals. Figure 2A shows the \(I_C\) traces generated by the application of five sequential pulses of ACh to an example WT cell. Figure 2B shows \(5 I_{ACh}\) traces obtained on an example mSOD1 cell showing a smaller peak amplitude. Two example \(I_{ACh}\) obtained from a WT and a mSOD1 cell are displayed in Fig. 2C; in both cases, current inactivation is likely due to the well-known desensitization of nicotinic receptors upon their exposure to ACh (35, 53). In 13 cells from 4 WT mice, \(I_{ACh}\) peak was 3.87 \pm 0.32 nA while in 43 cells from 9 mSOD1 mice was 1.16 \pm 0.06 nA, a 70% diminution (Fig. 2D).

\(I_K\) were investigated using a two-step depolarizing pulse protocol. First, a 10-ms predepolarization command to \(+30\) mV was given to allow Ca\(^{2+}\) entry and the activation of the calcium-dependent component of \(I_K\); then, a 400-ms depolarizing pulse to \(+120\) mV was applied to recruit both the Ca\(^{2+}\)-dependent and the voltage-dependent components of \(I_K\) (see protocol in Fig. 2E, top). The \(I_K\) traces of Fig. 2E were taken from a WT and mSOD1 cell subjected to the two-step depolarizing pulse protocol. Initially, there is a large outward current component that inactivates in \(\sim\)100 ms to a sustained plateau; these two components are due, respectively, to activation of \(I_{K(Ca)}\) and activation of \(I_{K(V)}\). \(I_{K(Ca)}\) are activated by the \([Ca^{2+}]_c\) transient generated by the prepulse and inactivate upon clearance of such transient to give rise to the sustained plateau, that is due to voltage-dependent K\(^+\) channels, which remain open for the entire 400-ms depolarizing pulse (52). Figure 2E shows that the \(I_{K(Ca)}\) and \(I_{K(V)}\) components of \(I_K\) were present in mSOD1 cells; they, however, were substantially lower than those recorded in the WT cell. These differ-

![Fig. 3](http://ajpcell.physiology.org/)

**Fig. 3.** Augmentation of the whole cell inward calcium current (\(I_{Ca}\)) and reduction of the whole cell inward sodium current (\(I_{Na}\)) in mSOD1 chromaffin cells, with respect to WT cells. To elicit calcium (\(I_{Ca}\)) and sodium (\(I_{Na}\)) currents cells were voltage clamped at \(-80\) mV and stimulated with 50-ms test depolarizing pulses to \(-10\) or 0 mV for \(I_{Ca}\), and with 10-ms test depolarizing pulses to \(-10\) mV for \(I_{Na}\). A: \(I_{Ca}\) traces of an example WT (dark trace) and mSOD1 (light trace) cells. B: averaged peak amplitude of \(I_{Ca}\) in WT and mSOD1 cells. C: \(I_{Na}\) traces of an example WT (dark trace) and mSOD1 (light trace) cells. D: averaged peak amplitude of \(I_{Na}\) in WT and mSOD1 cells. Data in B and D are means \pm SE of the number of cells and cultures shown in parentheses. **p < 0.01, with respect to WT cells (unpaired Student’s t-test).
ences are better illustrated in the bar diagram of Fig. 2F. Thus the averaged $I_{K(Ca)}$ component of 30 cells from five WT mice was $6.25 \pm 0.46 \text{nA}$ compared with $3.75 \pm 0.47 \text{nA}$ in 8 cells from mSOD1 mice, a 40% diminution; the values for the $I_{K(V)}$ component were $2.4 \pm 0.13 \text{nA}$ and $1.12 \pm 0.22 \text{nA}$, for WT and mSOD1 cells, respectively (a 53% diminution).

$I_{Ca}$ were generated by application of 50-ms test depolarizing pulses to 0 mV, as shown in the protocol in Fig. 3A, top; in this panel, two superimposed current traces from example WT and mSOD1 cells, with their initial rapidly inactivating $I_{Na}$ and later slow-inactivating $I_{Ca}$, are shown. The averaged peak amplitude of $I_{Ca}$ for WT and mSOD1 cells is shown in Fig. 3B, with $170 \pm 21.6 \text{pA}$ for WT cells and $270 \pm 28.1 \text{pA}$ for mSOD1 cells, a 37% increase. No differences of current kinetics between both cell types were observed.

Finally, $I_{Na}$ were generated with test depolarizing pulses to $-10 \text{mV}$, given at 10 s intervals. $I_{Na}$ traces obtained in WT and mSOD1 cells are shown in Fig. 3C; peak $I_{Na}$ amplitude was

![Graph showing elevations of the cytosolic Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{c}$) elicited by acetylcholine (ACh) or high potassium (K$^{+}$) in WT and mSOD1 chromaffin cells. Fura-2 AM loaded cells were perifused for 1 min with saline solutions containing 100 μM ACh (A) or 75 mM K$^{+}$ (low Na$^{+}$; B), as indicated by horizontal lines at bottom. A and B: overlapping [Ca$^{2+}$]$_{c}$ traces [expressed as arbitrary fluorescence units (AFU)] obtained in example WT and mSOD1 cells. Averaged pooled results of time to peak ($T_{\text{max}}$) of the [Ca$^{2+}$]$_{c}$ elevation (C), peak amplitude (D), and area (E) as indication of the total [Ca$^{2+}$]$_{c}$ transient of WT and mSOD1 cells. Data are means ± SE of the number of cells shown in parentheses in C, top, from at least 3 different cultures. ***P < 0.001 (one-way ANOVA test and Tukey post hoc analyses).](http://ajpcell.physiology.org/ by 10.220.33.1 on July 24, 2017)
20% smaller in the latter, respect to the former. Pooled data from 49 cells of 6 WT mice gave an $I_{Na}$ peak amplitude of $1.41 \pm 0.12 \, \text{nA}$, while in 25 cells from 6 mSOD1 mice $I_{Na}$ averaged $0.92 \pm 0.08 \, \text{nA}$, a 35% decrease (Fig. 3D).

Cytosolic calcium transients generated by cell depolarization with ACh or high-potassium in chromaffin cells from WT and mSOD1 mice. It is well established that ACh and high $K^+$ cause cell depolarization, enhanced Ca$^{2+}$ entry through voltage-

Fig. 5. Higher total catecholamine release in mSOD1 chromaffin cells compared with WT cells stimulated with ACh. A: example record obtained from a WT cell stimulated with 100 $\mu$M ACh for 1 min (horizontal bar at bottom). B: record obtained from an example mSOD1 cell similarly stimulated with ACh. C: cumulative secretion calculated at 5-s intervals in traces similar to those shown in A and B; the area of spikes is expressed in pC (ordinate) as a function of time (abscissa). D: secretion per stimulation period (integrated area of all spikes generated by the ACh pulse) in pC (ordinate). E: total number of spikes secreted per each ACh pulse (ordinate). F: quantal size of individual secretory events expressed in pC (ordinate). Data in C, D, and E are means $\pm$ SE of the number of cells and cultures shown in parentheses. Data in F are means $\pm$ SE of the number of individual spikes shown in parentheses; those spikes are from the experiments of D and E. *$P < 0.05$, ***$P < 0.001$, with respect to WT cells (unpaired Student’s t-test).
activated calcium channels (VACCs), elevation of $[\text{Ca}^{2+}]_c$, and the activation of the exocytotic release of catecholamine from chromaffin cells (1, 13, 17, 50). From a quantitative point of view, ACh and high K$^+$ trigger similar catecholamine release responses; however, the $[\text{Ca}^{2+}]_c$ transients generated by these two secretagogues are quite different (8). Therefore, we next explored the $[\text{Ca}^{2+}]_c$ transients generated by ACh and high K$^+$ in fura-2 AM loaded WT and mSOD1 chromaffin cells. After an initial 3-min period to get a stable resting baseline, cells were challenged with Tyrode solutions containing supramaximal depolarizing ACh concentrations (100 μM) or high K$^+$ (75 mM) during 1 min. This protocol was similar to that used to monitor the quantal release of catecholamine (see later). A given cell was stimulated with ACh or K$^+$ only once.

Example records of the time course of the $[\text{Ca}^{2+}]_c$ elevations produced by ACh and K$^+$ in WT and mSOD1 chromaffin cells are shown in Fig. 4, A and B, respectively. Although the ACh-evoked $[\text{Ca}^{2+}]_c$ transients were similar in WT and mSOD1 cells, the K$^+$-evoked $[\text{Ca}^{2+}]_c$ transients were notably higher in mSOD1 cells, as illustrated in the bar graphs of Fig. 4, C–E, showing that in WT and mSOD1 cells challenged with ACh time to peak (τ$_{\text{max}}$), peak amplitude, and area are similar for both types of cells. This was not the case for K$^+$; in mSOD1 cells, τ$_{\text{max}}$ is 20% lower with respect to WT cells. However, peak amplitude and area are 88 and 100% higher, respectively. Thus the K$^+$-elicited $[\text{Ca}^{2+}]_c$ transient had a faster activation as well as almost twofold enhanced $[\text{Ca}^{2+}]_c$ in mSOD1 with respect to WT cells. This agrees with the higher I$_c$ current observed in mSOD1 cells with respect to WT cells (Fig. 3B).

**Quantal catecholamine release responses triggered by ACh or high potassium in chromaffin cells from WT and mSOD1 mice.** To study the quantal release of catecholamine, all experiments began with an initial 5-min perfusion resting period with a standard Tyrode solution for adaptation of the targeted cell to its environment. None or few spontaneous amperometric secretory spikes were usually seen during this period. To study exocytosis, the basal Tyrode solution containing 2 mM Ca$^{2+}$ was quickly switched to another containing 100 μM ACh or 75 mM K$^+$ that bathed the cell for 1 min. This long stimulation period was used for two reasons: 1) we sought to get the maximal number of spikes to augment the power of statistical analysis of single exocytotic events; and 2) we also wished to explore potential differences in the time course of secretion linked to vesicle pool exhaustion and/or to the inactivation of VACCs (31, 69). A given cell from a culture dish was stimulated with ACh only once. About 75% of the tested mSOD1 cells and 85% of the WT cells responded to ACh.

Figure 5A shows the spike burst produced by ACh in an example WT cell; baseline was stable during the burst indicating the absence of overlapping spikes. Figure 5B shows a spike record from an example mSOD1 cell. Once more, in this cell no baseline elevation was produced although an initial high-frequency spike burst occurred; this was followed by a period with few spikes. Pooled data on cumulative secretion vs. time calculated at 5-s intervals from experiments similar to those shown in the example records of Fig. 5, A and B, were plotted in Fig. 5C. In both cell types, an initial faster rate of secretion during the first 10 s of the ACh pulse was followed by slower secretion rates that were slowly increasing along the next 50-s period. In mSOD1 cells, the initial secretion rate was 21.7% slower (time constant for activation, τ$_a = 13.7$ s) compared with WT cells (τ$_a = 11.27$ s). The integrated secretion (area in pC of all spikes secreted by each ACh pulse) is plotted in Fig. 5D; this total secretion was 46.6% higher in mSOD1 cells. However, in spite of this difference, the number of spikes per ACh stimulus was similar, 24.55 ± 2.22 for WT cells and 21.06 ± 1.22 for mSOD1 cells (Fig. 5E). The quantal size (single-vesicle content of catecholamine, Q) was 1.6-fold higher in mSOD1 cells (0.47 ± 0.03 pC) with respect to WT cells (0.31 ± 0.02 pC; Fig. 5F; Table 1). Thus, this higher total secretion with similar spike numbers could be explained by this greater quantal size of mSOD1 with respect to WT cells.

Concerning K$^+$ stimulation (75 mM, 1 min), most WT and mSOD1 (>95%) responded to this stimulus. The example WT and mSOD1 cells displayed in Fig. 6, A and B, generated an initial spike burst followed by more infrequent spikes, indicating a slower rate of secretion at later stages of the K$^+$ stimulation period. Of note was baseline elevation at the beginning of the K$^+$ pulse, suggesting the presence of overlapping spikes due to fast almost simultaneous exocytosis of docked vesicles of a readily releasable vesicle pool (RRP) (24, 46, 50). Cumulative secretion vs. time measured

### Table 1. Single spike kinetic parameters calculated from the secretory traces obtained in WT and mSOD1 chromaffin cells stimulated with ACh (100 μM) or K$^+$ (75 mM) for 1 min

<table>
<thead>
<tr>
<th>Cell Type (Stimulus)</th>
<th>Events (Cells)</th>
<th>Rise Rate, pA/ms</th>
<th>Decay 75–25%, ms</th>
<th>τ$1/2$, ms</th>
<th>I$_{\text{max}}$, pA</th>
<th>Q, pC</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (ACh)</td>
<td>2,034 (63)</td>
<td>23.75 ± 2.49</td>
<td>6.06 ± 0.38</td>
<td>5.71 ± 0.32</td>
<td>39.25 ± 3.17</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>mSOD1 (ACh)</td>
<td>1,357 (69)</td>
<td>15.21 ± 1.35</td>
<td>9.73 ± 0.65</td>
<td>8.86 ± 0.56</td>
<td>32.72 ± 2.08</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>WT (K$^+$)</td>
<td>1,848 (58)</td>
<td>17.19 ± 1.38</td>
<td>7.69 ± 0.43</td>
<td>6.94 ± 0.32</td>
<td>34.16 ± 1.88</td>
<td>0.4 ± 0.00</td>
</tr>
<tr>
<td>mSOD1 (K$^+$)</td>
<td>1,761 (74)</td>
<td>19.81 ± 1.31</td>
<td>8.11 ± 0.55</td>
<td>7.28 ± 0.58</td>
<td>36.69 ± 2.07</td>
<td>0.42 ± 0.03</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE with relative change of mSOD1 respect to wild type (WT; in mSOD1-ACh row) or relative change of K$^+$ respect to acetylcholine (ACh; in K$^+$ rows) given in parentheses. Individual spike data in each cell were analyzed and averaged; hence, final averaged data are expressed as the means of all cells (in parentheses). Rise rate was calculated with the slope of the ascending spike phase; decay time was calculated from the time between 75 and 25% of spike height in the descending spike phase; τ$1/2$ is the width of the spike at 50% of spike height; I$_{\text{max}}$ is the height of the spike; Q is the spike area, an indication of catecholamine vesicle content and release (quantal size). Chromaffin cells from WT and mSOD1 mice were compared for each single spike parameter (columns) using the nonparametric Mann-Whitney rank sum test. *P < 0.05, **P < 0.01, ***P < 0.001 in mSOD1 with respect to WT chromaffin cells for each stimulus; dP < 0.05, eP < 0.01, fP < 0.001 in mSOD1 with respect to WT chromaffin cells for each parameter. aData are presented as means ± SE with relative change of mSOD1 respect to wild type (WT; in mSOD1-ACh row) or relative change of K$^+$ respect to acetylcholine (ACh; in K$^+$ rows) given in parentheses. Individual spike data in each cell were analyzed and averaged; hence, final averaged data are expressed as the means of all cells (in parentheses). Rise rate was calculated with the slope of the ascending spike phase; decay time was calculated from the time between 75 and 25% of spike height in the descending spike phase; τ$1/2$ is the width of the spike at 50% of spike height; I$_{\text{max}}$ is the height of the spike; Q is the spike area, an indication of catecholamine vesicle content and release (quantal size). Chromaffin cells from WT and mSOD1 mice were compared for each single spike parameter (columns) using the nonparametric Mann-Whitney rank sum test. *P < 0.05, **P < 0.01, ***P < 0.001 in mSOD1 with respect to WT chromaffin cells for each stimulus; dP < 0.05, eP < 0.01, fP < 0.001 in K$^+$ with respect to ACh stimulus for each cell type (unpaired Student’s t-test in both cases).
at 5-s intervals was plotted in Fig. 6C. Unlike for ACh, the initial secretion was similar in WT cells (τa = 10.71 s) and mSOD1 cells (τa = 10.75 s). The two curves run in parallel and exhibited a low rate of increasing secretion. The integrated secretion (area in pC of all spikes secreted per each K⁺ pulse) is plotted in Fig. 6D; this secretion was similar in both cell types, around 12–13 pC. The total spike number (20.44 ± 1.98 for WT and 23.8 ± 1.76 for mSOD1; Fig. 6E) and the Q (0.4 ± 0.02 pC for WT and 0.42 ± 0.03 pC for mSOD1) were also similar (Fig. 6F and Table 1).
Spike frequency histograms counted at 2-s intervals during the 60-s stimulation periods with ACh or K$^+$ are shown in Fig. 7, A–D. The decay of the rate of spike frequency was best fitted to a single exponential in WT and mSOD1 cells. With ACh stimulation the time constant for inactivation ($\tau_i$) of spike frequency was 8.1 and 8.23 s, respectively, for WT and mSOD1 cells; in the case of K$^+$, $\tau_i$ amounted to 4.43 and 5.36 s for WT and mSOD1 cells, respectively (Fig. 7E). Thus the inactivation rate was 15 and 12% slower in mSOD1 cells, respectively, for ACh or K$^+$. Of note was the fact that spike frequency with ACh had a smaller plateau in mSOD1 cells (0.77%) than in WT cells (1.26%; Fig. 7, A and B). These differences were not apparent with K$^+$ (1.02 vs. 1.17%, for WT and mSOD1 cells, respectively; Fig. 7, C and D).

Fig. 7. Histograms of spike frequency vs. time analyzed on secretory traces obtained from WT and mSOD1 chromaffin cells stimulated with ACh or high potassium (K$^+$). A and B: frequency histograms of spike frequency (ordinates) vs. time (abscissa) challenged with ACh in WT and mSOD1 cells. C and D: frequency histograms of spike frequency (ordinates) vs. time (abscissa) challenged with 75 mM K$^+$ (low Na$^+$) in WT and mSOD1 cells. E: time constant for inactivation ($\tau_i$, ordinate in seconds) calculated from the decay of spike frequency of A–D. Data in panel E are means ± SE of the number of cells and cell cultures shown in parentheses in A–D. *P < 0.05 (unpaired Student’s t-test).
Types of secretory spike events in chromaffin cells from WT and mSOD1 mice. We next counted and analyzed the different types of spike events present in secretory traces generated by 1-min stimulation with ACh or K⁺. Figure 8 shows examples of the different subtypes of spikes analyzed, and Table 2 contains pooled results on the relative percentage of the different subtypes of spikes analyzed. With ACh stimulation, spikes with foot were 59.56 and 65.71% for WT and mSOD1 cells, respectively. With K⁺, the number of spikes with foot was similar in both cell types, ~70% (no statistically significant difference). Although an increase of spikes with foot seemed apparent in mSOD1 cells (65.71% with ACh and 73.1% with K⁺), these differences were only statistically significant with ACh compared with the values of WT cells (59.56% with ACh and 67.94% with K⁺). We also compared values of ACh and K⁺ stimuli, and differences were statistically significant both in WT and mSOD1 cells (Table 2).

Multiple spike events (overlapping spikes) indicate near-simultaneous quantal catecholamine release from vesicles belonging to a RRP. For instance, in a previous study we found as much as 30% overlapping spikes in cells from spontaneously hypertensive rats that likely had a larger RRP and hence they responded with massive exocytotic events upon their stimulation with ACh or K⁺; in contrast, cells from normotensive rats had only 15% of overlapping spikes (46). Compared with the rat, cells from WT mice had only 3.91% of multiple spike events when stimulated with ACh and around 5.69% when stimulated with K⁺ (Fig. 8, B and C; Table 2). Similar

Fig. 8. Types of secretory spike events in chromaffin cells from WT and mSOD1 mice stimulated with ACh or K⁺. Example spike events were selected from secretory traces from experiments of Figs. 5 and 6. A: spike with foot (left) and stand-alone spike (right) of a WT cell stimulated with ACh (100 μM). B: overlapping spikes in a WT cell stimulated with ACh. C: overlapping spikes and partial fusion pore (flicker) followed by a spike (full fusion pore) of a WT cell stimulated with K⁺ (75 mM, low Na⁺). D: spike with foot (left) and stand-alone spike of a mSOD1 cell stimulated with ACh (right). E: transient fusion pore (flicker) and overlapping spikes of a mSOD1 cell stimulated with ACh. F: flicker and overlapping spikes in a mSOD1 cell stimulated with K⁺. Quantitative pooled data on the frequency of these exocytotic spike events are summarized in Table 2.
values were obtained in cells from mSOD1 (4.15 and 5.09%, respectively) (Fig. 8, E and F; Table 2). Flickers are defined as small amounts of catecholamine release through a narrow fusion pore that opens transiently. Examples of these flickers are shown in Fig. 8, B and C (WT cells stimulated, respectively, with ACh or K+/H11001) and in Fig. 8, E and F (mSOD1 cells stimulated, respectively, with ACh or K+/H11001). We found that 69.8% of ACh-elicited secretory responses from WT cells had at least one flicker; this was higher than the value of 60.3% obtained when these cells were stimulated with K+/H11001. Of those, 6.9 and 7.52% of the total spike number were flickers. On the other hand, 69.6 and 82.4% of mSOD1 cells stimulated with ACh and K+/H11001 had one flicker at least, and flickers were 8.84 and 8.13% of the total spike number, respectively (Table 2).

Kinetic analysis of single exocytotic events occurring in the secretory responses triggered by ACh and high potassium in chromaffin cells from WT and mSOD1 mice. Figure 9A shows a schematic model of an amperometric secretory spike with foot, indicating the parameters that we measured according to the criteria established by Machado et al. (42) for good practices in single-cell amperometry. Four types of comparative analyses were performed: 1) WT (2,034 spikes, 63 cells) vs. mSOD1 cells (1,357 spikes from 69 cells) stimulated with ACh; 2) WT (1,848 spikes, 58 cells) vs. mSOD1 cells (1,761 spikes, 74 cells) stimulated with K+/H11001; 3) mSOD1 (ACh) vs. WT (ACh); and 4) mSOD1 (K+/H11001) vs. WT (K+/H11001). Data were obtained through the manual analysis of secretory event traces obtained from experiments shown in Figs. 5, A and B (ACh), and 6, A and B (K+/H11001), counting the different types of events as displayed in Fig. 8. *P < 0.05 in mSOD1 with respect to WT chromaffin cells for each stimulus; †P < 0.01 in K+/H11001 with respect to ACh stimulus for each cell type (unpaired Student’s t-test in both cases).

Table 2. Characteristics of exocytotic spike events in WT and mSOD1 chromaffin cells stimulated with ACh (100 μM) or K+ (75 mM) for 1 min

<table>
<thead>
<tr>
<th>Cell Type (Stimulus)</th>
<th>Events (Cells)</th>
<th>Spikes with Foot, %</th>
<th>Multiple-Spike Events, %</th>
<th>Flickering, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (ACh)</td>
<td>2,034 (63)</td>
<td>59.56 ± 2.17</td>
<td>3.91 ± 0.46</td>
<td>6.9 ± 0.88</td>
</tr>
<tr>
<td>mSOD1 (ACh)</td>
<td>1,357 (69)</td>
<td>65.71 ± 1.91* (10.33% higher)</td>
<td>4.15 ± 0.55 (6.14% higher)</td>
<td>8.4 ± 0.94 (28.12% higher)</td>
</tr>
<tr>
<td>WT (K+)</td>
<td>1,848 (58)</td>
<td>67.94 ± 2.28* (14.08% higher)</td>
<td>5.69 ± 0.74 (45.52% higher)</td>
<td>7.52 ± 0.72 (8.99% higher)</td>
</tr>
<tr>
<td>mSOD1 (K+)</td>
<td>1,761 (74)</td>
<td>73.12 ± 1.72* (11.27% higher)</td>
<td>5.09 ± 0.57 (22.65% higher)</td>
<td>8.13 ± 0.91 (8.03% lower)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE with relative change of mSOD1 respect to WT (in mSOD1-ACh row) or relative change of K+ respect to ACh (in K+ rows) given in parentheses. Data were obtained through the manual analysis of secretory event traces obtained from experiments shown in Figs. 5, A and B (ACh), and 6, A and B (K+), counting the different types of events as displayed in Fig. 8. *P < 0.05 in mSOD1 with respect to WT chromaffin cells for each stimulus; †P < 0.01 in K+ with respect to ACh stimulus for each cell type (unpaired Student’s t-test in both cases).

Fig. 9. Prototype spike shapes obtained from averaged pooled data on the kinetic parameters of individual exocytotic spike events taken from experiments of Figs. 5 and 6 and summarized in Table 1, done in chromaffin cells from WT and mSOD1 mice. A: spike model showing the kinetic parameters calculated for the foot (Table 3) and the spike (Table 1). B: superimposed averaged spikes for WT (continuous line) and mSOD1 cells (discontinuous line) stimulated with ACh (100 μM). C: superimposed averaged spikes for cells of WT (continuous line) and mSOD1 (discontinuous line) stimulated with K+ (75 mM, low Na+). D: superimposed averaged spikes for WT cells stimulated with ACh (continuous line) or K+ (discontinuous line). E: superimposed averaged spikes for mSOD1 cells stimulated with ACh (continuous line) and K+ (discontinuous line). F: overlapping averaged spikes from chromaffin cells from APP/PS1 mouse model of AD [discontinuous line, taken from de Diego et al. (14)] and an ALS mouse model (continuous line, from this study).
WT and mSOD1 cells directly depolarized with K+; 3) WT cells stimulated with ACh vs. K+; and 4) mSOD1 cells stimulated with ACh vs. K+.

All parameters studied on WT and mSOD1 cells indirectly depolarized with the physiological neurotransmitter ACh exhibited statistically significant differences (Table 1). Thus, compared with WT, mSOD1 cells had the following changes: 35.94% lower rise rate, 60.7% longer decay time, 55.3% higher half-amplitude time (half-width, t1/2), 16.62% lower amplitude (Imax), and 52.24% higher Q. Averaged spikes with these parameters resulted in a narrower but faster spike for WT cells, compared with a slower spike with higher Q for mSOD1; this indicated a slower but higher exocytotic release of catecholamine per single vesicle in the latter, compared with the former as indicated in the averaged overlapping spikes of Fig. 9B.

Surprisingly, all kinetic parameters of spikes recorded from WT and mSOD1 cells directly depolarized with K+ had similar values (Table 1). This indicated that the averaged exocytotic spike did not differ between both cell types when they were directly depolarized with K+, as the overlapping averaged spikes indicate in Fig. 9C.

We also noted some kinetic differences in the spikes generated by ACh or K+ stimulation of WT cells. Thus, compared with ACh-stimulated cells (indirect depolarization through intermittent APs), the K+-stimulated cells (continuous direct depolarization) had 27.62% lower rise rate, 27.05% higher decay time, 21.57% higher t1/2, 12.97% lower Imax, and 28.77% higher Q. The overlapping averaged spikes of Fig. 9D indicate a faster but smaller release of catecholamine when WT cells were stimulated with ACh, compared with K+.

Finally, we compared the kinetic parameters of the secretory events recorded in mSOD1 cells stimulated with ACh or K+, which showed only minor differences. For instance, compared with ACh, the K+ elicited spikes had 30.24% higher rise rate, 16.43% lower decay time, and 17.81% lower t1/2; the rest of the parameters were similar (no statistically significant differences). Overlapping averaged spikes indicated that the K+ elicited secretory response was slightly faster but with Q similar to ACh (Fig. 9E).

Spikes with foot were slightly more frequent (10.33%) in ACh-stimulated mSOD1 cells, compared with WT cells (Table 3). Foot duration was 30.8% longer and had 22.95% lower amplitude in mSOD1 cells compared with WT cells. No statistically significant differences were observed between both cell types upon their stimulation with K+ (Table 3).

Immunoblot analysis of the expression of proteins of the exocytic machinery and the α5-subunit of nicotinic receptors in WT and mSOD1 mice. The detailed analysis of the kinetics of amperometric secretory spikes is contributing to a better understanding of the molecular mechanisms through which the different proteic components of the exocytotic machinery regulate the last steps of exocytosis and hence of neurotransmitter release. In fact, chromaffin cells from transgenic mice with genetic manipulations of SNARE proteins have extensively been used to explore specific mutations of those proteins on the formation and expansion of the fusion pore (66). We therefore thought it of interest to explore the expression of three SNARE proteins and synaptotagmin in the adrenal medulla, brain cortex, and hippocampus, as well as the spinal cord of WT and mSOD1 mice.

Figure 10 displays the relative densities of SYT7 (A), STX1 (B), SNAP25 (C), and VAMP2 (D) in the adrenal medulla of WT and mSOD1 mice. We found no statistical differences in the relative expression level of SYT7, STX1, SNAP25, and VAMP2 in the adrenal medulla between WT and mSOD1; a decreased 20% expression of VAMP2 was at the limit of statistical significance. We also monitored the expression of the NACHRA3 known to be expressed at high density in chromaffin cells and to mediate the catecholamine release response elicited by ACh (7). Figure 10E shows that the NACHRA3 was similarly expressed in the adrenal medullary tissue of WT and mSOD1 mice.

We also tested the expression of proteins in the brain cortex, hippocampus, and spinal cord of WT and mSOD1 mice; the results are summarized in Fig. 11. Concerning WT and mSOD1 tissues, we found no differences in the expression of SYT1, STX1, VAMP2, and NACHRA3 in the cortex, hippocampus, and spinal cord (Fig. 11; A, B, D, and E). A 25% decrease of SNAP25 expression was found in the spinal cord of mSOD1 mice, with respect to WT mice (Fig. 11C; P < 0.05).

Motor deficits of mSOD1 with respect to WT mice. In this study we used WT and mSOD1 mice of ages between P90 and P130 days. At these ages the mutated mice show adult ALS onset (25, 29) and significant loss of vulnerable spinal motor neurons (10). Although motor impairment of mSOD1 at these ages is well documented, we, however, explored the motor activity of the WT and mSOD1 mice used here, by means of the Rota-Rod test, which assesses motor coordination and balance, as described by Jones and Roberts (34). Animals were placed on the rolling rod (32-mm diameter) with an initial
Fig. 10. Relative expression of proteins of the exocytotic machinery and the α3-subunit of nicotinic receptors in adrenal medullary tissue from WT and mSOD1 mice, monitored with Western blot. An immunoblot from a representative experiment is shown at top of A–D. Top bands correspond to synaptotagmin 7 (SYT7; A), syntaxin 1 (STX1; B), synaptosomal-associated protein 25 1 (SNP25; C), vesicle-associated membrane protein 2 (VAMP2; D), and neuronal ACh receptor subunit α3 (NACHRA3; E); bottom bands correspond to control β-actin (ACTB). Bar diagrams represent pooled averaged results of adrenal glands from WT and mSOD1 mice. The relative band densities were calculated as ratios between the density of each band protein and its corresponding ACTB band, within each individual immunoblot. Values are means ± SE. There are not significant differences between WT and mSOD1 mice (unpaired Student’s t-test).
The speed of 8 rpm and an increase of 1 rpm each 8 s until they fell down; we made 2 measures of 10 repetitions: the training day (Trd) and the test day (Td), in 2 consecutive days, comparing WT and mSOD1 mice at P90 and P130 ages, as shown in Fig. 12. mSOD1 mice show a significant decrease in the time they stay on Rota-Rod compared with WT mice in both P90 (Fig. 12A, $P < 0.05$) and P130 (Fig. 12B, $P < 0.001$) as well as in both, Trds and Tds. Also, when comparing Trd and Td we saw...
mSOD1 mice were not able to improve the time they stay on the rod at both ages, in contrast with WT mice ($P < 0.01$). It is remarkable that mSOD1 mice show a significant 56% decrease of the time to fall in P130 compared with P90 ($P < 0.05$), while WT mice show similar times (Fig. 12C).

**DISCUSSION**

In this study, pronounced alterations in cell excitability and ion currents, as well as in the kinetics of the exocytotic fusion pore, have been found to occur in chromaffin cells from mSOD1, compared with WT mice. One of the most notable was the almost disappearance of spontaneous APs, an intrinsic property of chromaffin cells (13). The resting $V_m$ in bisected mouse adrenal chromaffin cells using intracellular recordings in situ is 54.3 mV (49). Using the patch-clamp technique we have found here that WT and mSOD1 had somehow more hyperpolarized resting $V_m$, namely $-68$ and $-63$ mV, respectively. Considering that the resting $V_m$ was at the same level in both cell types, the drastic difference in spontaneous firing may find an explanation in the depressed inward $I_{Na}$ as well as in the outward $I_{K(Ca)}$ and $I_{K(V)}$, in mSOD1, with respect to WT cells, since these currents are known to contribute to the generation of APs in chromaffin cells (3, 5, 38, 45, 49).

Our results on depressed ion currents agree with those of the study of Boutahar et al. (4) dealing with the transcriptional profile of cortical neurons from E14 embryos of WT and mSOD1 mice. In mSOD1 neurons, the expression of Na$^+$ channel type VIIα (SNC7A) is depressed 7.2-fold and that of large-conductance Ca$^{2+}$-dependent K$^+$ channel (KCNA1, BK channel) is depressed by 21-fold with respect to WT neurons. Also, in ALS patients immunoreactivity of Kv1.2 channels is markedly reduced in the ventral root (65). Furthermore, SH-SY5Y cells expressing the mutated SOD1$^{G93A}$ have a decreased $I_{Na}$ (76). This depressed gene expression in cortical neurons supports our conclusion that the depressed excitability of mSOD1 chromaffin cells is likely due to poorer expression of Na$^+$ and K$^+$ channels, thus explaining the decreased $I_{Na}$ and $I_K$ we found in these cells. A toxic gain of function has been considered the cause of motoneuron degeneration on mice overexpressing human mSOD1 (29). This hypothesis received support from a study on PC12 cells transfected with SOD1$^{G93A}$ that exhibit an increased production of hydroxyl radicals and an enhanced rate of apoptotic cell death (40).

Bulk secretion of catecholamine, measured as the total number of individual spikes counted during the 1-min stimulation with ACh, was similar in both cell types. In principle, this agrees with the similar [Ca$^{2+}$], transients elicited by ACh, in spite of the fact $I_{Ca}$ was mildly enhanced in mSOD1 chromaffin cells, in agreement with a previous study showing that N-type VACCs were overexpressed in cortical neurons of mSOD1 mouse embryos (56). However, if bulk secretion is measured as a summatory of all spike areas, it came about that mSOD1 cells secreted 46.6% more catecholamine than WT cells. This may be explained if the 52.24% higher $Q$ of individual spikes is taken into account. Similar [Ca$^{2+}$], transients and secretion elicited by ACh are not in agreement with the fact that $I_{ACH}$ was 70% smaller in mSOD1 with respect to WT cells. Because the protein expression of the α3-subunit of nicotinic receptors is similar in both cell types, the 70% depressed $I_{ACH}$ in mSOD1 cells may be due to an altered receptor function. Compensatory overexpression of other nicotinic receptor subtypes may also have occurred.

An interesting difference was found in the kinetic parameters of the exocytotic fusion pore, averaged from calculations done in thousands of individual secretory amperometric spikes. These differences are as follows (mSOD1 vs. WT cells): 35.94% lower rise rate, 60.7% higher decay time, 55.3% higher $t_{1/2}$, and 52.24% higher $Q$. This means that the expansion of the fusion pore is slower, the release of vesicle contents takes longer, and the release of catecholamine per vesicle is higher. In other words, considering the 1-min period of cell stimulation with ACh, the 21 vesicles that undergo exocytosis in mSOD1 chromaffin cells release 50% more catecholamine than the 24–25 vesicles of WT cells but at a lower rate.

Probably, the more striking finding in this study is related to the fact that the drastic differences in the secretion parameters found between WT and mSOD1 cells when stimulated with ACh were not present when cells were stimulated with high K$^+$. For many years, ACh, high K$^+$, barium (Ba$^{2+}$), and several other secretagogues have been used to trigger exocytosis and even to analyze the kinetics of the fusion pore (43,
Ca²⁺ concentrations at subplasmalemmal sites while population M2 could explain the paradox of enhanced K⁺ responses elicted by sustained depolarization with K⁺ in a manner that considerably differs from the responses elicited by high K⁺ /H11001. This indicates that an altered neurotransmission is present at the adrenal medulla of mSOD1 mice. Relevant to this study is that the changes in the kinetics of the exocytotic fusion pore in chromaffin cells from the mSOD1 mouse model of ALS, respect to WT mice, when physiologically challenged with ACh. A slower fusion pore opening, expansion, and closure are likely due to a pronounced reduction of cell excitability and ion currents driving APs in mSOD1 chromaffin cells. These changes may help to better understand human ALS pathogenesis and to inspire novel biological targets for the eventual development of a medicine to treat patients suffering of intractable ALS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

ALTERED FUSION PORE KINETICS IN THE SOD1G93A MICE


