Synaptotagmin-1 and -7 are functionally overlapping Ca\textsuperscript{2+} sensors for exocytosis in adrenal chromaffin cells

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Synaptotagmin-1, the canonical isoform of the synaptotagmin family, is a Ca\textsuperscript{2+} sensor for fast synchronous neurotransmitter release in forebrain neurons and chromaffin cells. Even though deletion of synaptotagmin-1 abolishes fast exocytosis in chromaffin cells, it reduces overall secretion by only 20% because of the persistence of slow exocytosis. Therefore, another Ca\textsuperscript{2+} sensor dominates release in these cells. Synaptotagmin-7 has a higher Ca\textsuperscript{2+} affinity and slower binding kinetics than synaptotagmin-1, matching the proposed properties for the second, slower Ca\textsuperscript{2+} sensor. Here, we examined Ca\textsuperscript{2+}-triggered exocytosis in chromaffin cells from KO mice lacking synaptotagmin-7, and from knockin mice containing normal levels of a mutant synaptotagmin-7 whose C\textsubscript{2B} domain does not bind Ca\textsuperscript{2+}. In both types of mutant chromaffin cells, Ca\textsuperscript{2+}-triggered exocytosis was decreased dramatically. Moreover, in chromaffin cells lacking both synaptotagmin-1 and -7, only a very slow release component, accounting for ∼30% of WT exocytosis, persisted. These data establish synaptotagmin-7 as a major Ca\textsuperscript{2+} sensor for exocytosis in chromaffin cells, which, together with synaptotagmin-1, mediates almost all of the Ca\textsuperscript{2+} triggering of exocytosis in these cells, a surprising result, considering the lack of a role of synaptotagmin-7 in synaptic vesicle exocytosis.

amperometry | calcium-binding protein | capacitance | dense core vesicle | fusion

Release of neurotransmitters and hormones is triggered by Ca\textsuperscript{2+} influx, which leads to exocytosis of synaptic or secretory vesicles. Synaptotagmins constitute a family of proteins containing a single transmembrane region and two Ca\textsuperscript{2+}- and phospholipid-binding C\textsubscript{2} domains. Synaptotagmin-1, the prototype synaptotagmin, functions as a vesicular Ca\textsuperscript{2+} sensor for fast exocytosis in forebrain neurons and chromaffin cells (1–5). In the absence of synaptotagmin-1, the fastest phase of exocytosis is abolished; however, a slower form of exocytosis persists (3, 5–7). In chromaffin cells, this slower form seems to represent release from a distinct “slowly releasable” pool (SRP) (or state) of vesicles that have not yet reached the “readily releasable” state (readily releasable pool or RRP) (3, 8). In fact, exocytosis in chromaffin cells occurs predominantly via the slower pathway when triggered by a step elevation of [Ca\textsuperscript{2+}]. As a result, deletion of synaptotagmin-1 leads to only a ∼20% reduction in the amount of exocytosis measured after 5 s (3, 8).

The mild effect of the synaptotagmin-1 deletion on overall exocytosis in chromaffin cells raises important questions. This observation supports the notion that synaptotagmins might not be important for regulating overall release but only for timing the release relative to the Ca\textsuperscript{2+} trigger (6). An alternative view holds that synaptotagmins do actively trigger release but that there is a second synaptotagmin Ca\textsuperscript{2+} sensor in chromaffin cells that is responsible for slower forms of exocytosis (1). Of the 15 mammalian synaptotagmins, only 8 display Ca\textsuperscript{2+}-dependent lipid binding (synaptotagmin-1, -2, -3, -5, -6, -7, -9, and -10). Recent studies have identified synaptotagmin-1, -2, and -9 as alternative and parallel Ca\textsuperscript{2+} sensors in synaptic exocytosis (9), and synaptotagmin-1 and -9 have been shown to account for essentially all release in PC12 cells, a phaeocytochroma cell line derived from the adrenal medulla (10, 11). However, synaptotagmin-2 and -9 are not expressed in chromaffin cells (9, 12, 13), and even though kinetic differences between synaptotagmin-1, -2 and -9 isoforms are detectable (8, 9), they are all relatively fast acting isoforms (14) and thus cannot explain radically slower forms of exocytosis. Thus, the identity of the slow Ca\textsuperscript{2+} sensor, which is crucial for exocytosis in chromaffin cells, remains obscure.

Synaptotagmin-7 is unusual in that it is ubiquitously expressed early in development but restricted to secretory cells with Ca\textsuperscript{2+}-regulated exocytosis in the first postnatal weeks (15). As a potential Ca\textsuperscript{2+} sensor for slower forms of exocytosis, synaptotagmin-7 is of special interest because it shows maximal Ca\textsuperscript{2+}-dependent phospholipid binding in the low micromolar Ca\textsuperscript{2+} range (∼3 μM; (16)), which fits well with the higher apparent Ca\textsuperscript{2+} affinities of slower exocytosis in chromaffin cells (17). In addition, synaptotagmin-7 displays slower lipid-unbinding kinetics than synaptotagmin-1 (14) and can confer Ca\textsuperscript{2+} dependence on SNARE-dependent vesicle fusion (18), consistent with a function as a slow sensor.

Several studies suggested a role for synaptotagmin-7 in Ca\textsuperscript{2+}-dependent exocytosis of secretory vesicles and lysosomes (19, 20), but results are contradictory as to whether synaptotagmin-7 is required for lysosome exocytosis per se (21, 22) or for restricting fusion pore expansion (23). In PC12-cells, overexpressed exogenous synaptotagmin-7 can stimulate exocytosis (24–26), but the function of endogenously expressed synaptotagmin-7 in these cells is questioned by the finding that synaptotagmin-1 and synaptotagmin-9 may account for all exocytosis (11). Moreover, the question whether Ca\textsuperscript{2+} binding to endogenous synaptotagmin-7 is necessary for its function, as would be expected for a protein acting as a Ca\textsuperscript{2+} sensor, has not been addressed. Furthermore, the finding that synaptotagmin-7 is not required for any form of synaptic vesicle exocytosis (27), despite its synaptic localization (19) and the effect of its overexpression on synaptic vesicle recycling (28), raises doubts about the possible Ca\textsuperscript{2+}-sensor function of synaptotagmin-7 in exocytosis. Thus, we have in the present study examined the possible role of

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synaptotagmin-7 in chromaffin granule exocytosis. Our results unequivocally establish that synaptotagmin-7 is an essential Ca\(^{2+}\)-sensor for this type of exocytosis, revealing an unexpected specialization of different synaptotagmin isoforms for different types of exocytosis.

**Results**

Immunoblotting results indicated that chromaffin cells in mice express synaptotagmin-1 and -7, but appear to lack synaptotagmin-2, -9 (Fig. 1A) and (9, 29). Thus, we analyzed Ca\(^{2+}\)-triggered exocytosis in chromaffin cells from mutant mice that lack synaptotagmin-7 (KO), either alone or in conjunction with a deletion of synaptotagmin-1, or that contain normal levels of a mutant synaptotagmin-7 containing amino acid substitutions in its C\(2B\)-domain that prevent Ca\(^{2+}\)-binding to this domain (27).

Depolarization-Induced Release Is Moderately Impaired by Deletion of Synaptotagmin-7. We first investigated basic properties of exocytosis in chromaffin cells obtained from young adult (1–2-months of age) synaptotagmin-7 KO (−/−) mice and their WT (WT, +/+ ) littermates. After establishment of a whole-cell recording configuration, chromaffin cells were stimulated by a train of depolarization pulses, and exocytosis was monitored by membrane capacitance measurements and amperometry (Fig. 1B). In synaptotagmin-7-deficient cells, exocytosis was moderately depressed when assayed by capacitance measurements (−54%; \(P = 0.007\), Mann–Whitney test), and more dramatically depressed when measured by amperometry (−52%; \(P = 0.007\)). Measurements of the Na\(^{+}\) and Ca\(^{2+}\) currents did not reveal significant differences between synaptotagmin-7 KO and WT mice (Fig. 1C and data not shown). However, Ca\(^{2+}\) measurements indicated that the [Ca\(^{2+}\)] concentration was elevated to slightly higher levels in KO cells (\(P < 0.05\)), which could have obscured the effect of the deletion of synaptotagmin-7.

Deletion of Synaptotagmin-7 Affects the Extent and Kinetics of Exocytosis. In a second round of experiments, we elicited exocytosis by flash photolysis of caged Ca\(^{2+}\), which causes a step-like, homogeneous increase of [Ca\(^{2+}\)]. In WT cells, this stimulation protocol resulted in a fast increase in capacitance (the secretory burst), which corresponds to the fusion of two pools of releasable vesicles, the slowly and the readily releasable pool (Fig. 2A). A later, much slower part of the capacitance increase—the sustained component—is triggered by release of granules that are being primed during the stimulation period. The time integral of the amperometric current generally mirrored the capacitance signal, except for a diffusional delay (Fig. 2A Lower).

We found that in synaptotagmin-7-deficient cells, both the exocytotic burst and the sustained component were reduced (Fig. 2A and D–E) by ÷ 50%. Importantly, postflash [Ca\(^{2+}\)] were closely matched between KO and WT cells (Fig. 2A Top), which explains the clearer phenotype of the KO during these measurements. The relationship between the cubic root of the amperometric charge and the square root of the capacitance change was approximately linear in both cases (KO and WT) with tightly overlapping regression lines (Fig. 2B). This finding indicates no difference in the released amount of catecholamines per unit area of vesicle membrane after removal of synaptotagmin-7. These data agree with findings in the synaptotagmin-1 KO (8).

To investigate the kinetics of exocytosis, we examined the mean capacitance traces from the KO and WT cells within the first 500 ms after the flash (Fig. 2C). The capacitance traces followed each other during the first ÷ 200 fF increase, after which time the KO trace deviated. Consequently, if we normalize the traces to the same amplitude at 0.5 s after the flash, the KO leads the WT trace (dotted line in Fig. 2C), indicating relative faster release kinetics in the KO. We next estimated the release kinetics by fitting individual capacitance traces with a sum of exponentials. The amplitudes represent the vesicle pool sizes, whereas the time constants identify their fusion kinetics. We found that in synaptotagmin-7-deficient cells, the fast burst was significantly smaller (−58%, \(P < 0.0001\), Fig. 2D), its time constant slightly faster (−25%, \(P = 0.0009\), Fig. 2F), and the delay between the flash stimulation and the start of the capacitance increase shorter (−44%, \(P = 0.0003\), Fig. 2F) than in WT cells. The slow burst was significantly smaller in KO cells (−40%, \(P = 0.0065\), Fig. 2D), but the fusion time course was not affected (Fig. 2F).

The apparently faster fusion kinetics of the RRP in the absence of synaptotagmin-7 allows at least two alternative interpretations: Either the KO of synaptotagmin-7 causes some vesicles to fuse with bona fide faster kinetics, or the decrease in the size of the RRP has uncovered a subpool with slightly faster kinetics, which is also present in WT cells. For two reasons, we favor the second interpretation. First, the capacitance trace of...
the KO followed the WT trace for the first ~20 fF (Fig. 2D), corresponding to the size of the RRP in KO cells (Fig. 2D). Second, we found that larger RRP amplitudes correlated weakly but significantly with larger time constants in both KO and WT cells (Fig. 2G). Therefore, WT cells with smaller RRP sizes also display a slightly faster kinetics. We interpret this as evidence for heterogeneity within the RRP, where a small, faster pool appears to be disguised by a larger and somewhat slower pool in cells with a large RRP size. In synaptotagmin-7 KO cells, only the fastest subpool of the RRP is present.

**Deletion of Synaptotagmin-7 May Impair the Refilling of the Releasable Pools.** Deletion of synaptotagmin-7 reduced the sustained component of release by 40% \((P = 0.0012;\) Fig. 2A and E). This near-linear component reports on the vesicle priming process, which is much slower than the triggering step at these \(\text{Ca}^{2+}\) concentrations. Examination of the relative amplitudes of two consecutive flashes applied at a 90-sec interval also provides useful information about the refilling of the releasable pools. In WT cells, the second flash elicited elicited smaller responses \([77\% of the first flash amplitude, \(P = 0.002; n = 51, N = 6, (n = number of cells recorded; \(N = number of mice used for each experimental series)]\). This secretory depression was, however, more pronounced in absence of synaptotagmin-7 \((48\% of the first burst amplitude, \(P = 0.002; n = 51, N = 6, (n = number of cells recorded; \(N = number of mice used for each experimental series)]\). This observation suggests that deletion of synaptotagmin-7 impaired releasable pools refilling between stimuli.

**\(\text{Ca}^{2+}\) Sensitivity of Exocytosis in the Low-Micromolar Range Is Not Altered by Deletion of Synaptotagmin-7.** Using flash stimulation, we showed that deletion of synaptotagmin-7 modifies exocytosis at intermediate \((20–30 \mu\text{M})\ \text{Ca}^{2+}\) levels. Because synaptotagmin-7 is proposed to be a high-affinity \(\text{Ca}^{2+}\) sensor, we next studied the \(\text{Ca}^{2+}\) dependence of exocytosis rates in the low-micromolar range \((0.3–10 \mu\text{M})\) using \(\text{Ca}^{2+}\) ramps created by gradual \(\text{Ca}^{2+}\) release from caged \(\text{Ca}^{2+}\) (2).

We first examined \([\text{Ca}^{2+}]-threshold values, i.e., the \([\text{Ca}^{2+}]+at which exocytosis shows maximal acceleration (Fig. 3A)). This value is increased after mutation of synaptotagmin-1, which lowers \(\text{Ca}^{2+}\)-affinity (2). We found no significant change in this parameter in synaptotagmin-7 KO mice, which is a first indication of no major change in the \(\text{Ca}^{2+}\) sensitivity of release (Fig. 3B). We next calculated the rate of capacitance change as a function of \([\text{Ca}^{2+}]+and normalized the rates to the remaining pool size (Fig. 3C). This normalization leads to the identification of the pool size-independent fusion rate constant. No difference between KO and WT cells was found (Fig. 3C). Thus, deletion of synaptotagmin-7 does not profoundly modify the exocytotic rate constant in the low-micromolar \(\text{Ca}^{2+}\) concentration range.
Ca\textsuperscript{2+} Binding to the C\textsubscript{2}B Domain of Synaptotagmin-7 Is Essential for Triggering Exocytosis. Because deletion of synaptotagmin-7 alters several phases of exocytosis, we next asked whether these effects depend on Ca\textsuperscript{2+} binding. Thus, we analyzed exocytosis from synaptotagmin-7 knockin (KI) mice, which express normal levels of synaptotagmin-7 with a mutated C\textsubscript{2}B-domain (27). Importantly, flash experiments in homozygous KI (\(-/-\)) mice, which WT (+/+ ) littermates closely reproduced the phenotype identified in synaptotagmin-7 KO mice (Fig. 4). Overall, exocytosis was reduced in the KI (Fig. 4A), because of changes in both the burst phase and the sustained phase of release. Kinetic analysis showed that the fast burst was significantly reduced in amplitude (Fig. 4B), but displayed faster kinetics (Fig. 4C), as in synaptotagmin-7 KO mice. Other than in the KO mice, the slow burst was not significantly changed (Fig. 4B and C); however, a tendency to a reduction was still seen. Finally, the sustained component of release was decreased (Fig. 4B). Overall, these findings demonstrate that the main changes in the synaptotagmin-7 KO are reproduced when synaptotagmin-7 is unable to bind Ca\textsuperscript{2+} by its C\textsubscript{2}B domain, indicating that synaptotagmin-7 acts as a Ca\textsuperscript{2+} sensor during exocytosis of dense-core vesicles (see also Table 1).

Synaptotagmins-1 and -7 Functionally Cooperate in Exocytosis. Searching for a second Ca\textsuperscript{2+} sensor in chromaffin cell exocytosis was motivated by the modest effect of deleting synaptotagmin-1, which only leads to the elimination of the fast burst of release (3, 8). To test whether synaptotagmin-7 is responsible for the residual slower exocytosis in synaptotagmin-1 deficient chromaffin cells, we generated a double-KO mouse line. Because synaptotagmin-1 null mice are not viable, we used chromaffin cells isolated from embryonic day (E)18 embryos. Embryonic chromaffin cells might, in certain cases, deviate from adult cells by expressing different protein isoforms (30). We therefore repeated the experiments with synaptotagmin-7 KO using embryonic mice and found qualitatively the same phenotype as in adult cells [supporting information (SI) Fig. 6]. Next, we prepared chromaffin cells from E18 embryos that resulted from crossings of mice that were synaptotagmin-1 heterozygotes and synaptotagmin-7 hetero- or homozygotes. Flash stimulation of control chromaffin cells (heterozygous for synaptotagmin-7) resulted in normal fast exocytosis (Fig. 5, black trace). Note that exocytosis in these cells (Fig. 5) were similar to synaptotagmin-7 WT embryonic cells (compare SI Fig. 6), indicating that the heterozygous condition was without effect. In agreement with previous data, deletion of synaptotagmin-1 (in the synaptotagmin-7 heterozygous background) resulted in the elimination of fast-burst exocytosis, whereas the slow exocytotic burst and the sustained component persisted (3, 8) (Fig. 5, red trace). Strikingly, deletion of synaptotagmin-7 in the synaptotagmin-1 KO background almost completely eliminated slow-burst exocytosis (Fig. 5, gray trace). The remaining exocytosis amounted to only 20–30% of WT secretion and was almost linear in shape, without any signs of an initial faster (burst-like) phase.

Because of the low levels of exocytosis, kinetic analysis of
individual recordings was not possible in the double-KO cells. Instead, we fitted the averaged double-KO capacitance trace. Exocytosis in the double KO could be described by a sustained component (50% of the control), and a very small slow burst component (13% of control cells; Table 1) but without the use of a fast burst component (as in synaptotagmin-1 KO cells). This confirms that synaptotagmin-7 is responsible for the slower phase of the exocytotic burst in synaptotagmin-1 KO chromaffin cells. Thus, synaptotagmin-1 and synaptotagmin-7 display context-dependent redundancy as Ca$^{2+}$ sensors for exocytosis in chromaffin cells.

**Discussion**

We have investigated the role of synaptotagmin-7 in Ca$^{2+}$-triggered exocytosis using mutant mice that either entirely lack synaptotagmin-7 expression or contain normal levels of a mutant synaptotagmin-7 that is unable to bind Ca$^{2+}$ via its C2B domain (27). Our results establish that synaptotagmin-7 is important for exocytosis in chromaffin cells and that synaptotagmin-1 and -7 together account for most of the Ca$^{2+}$-triggered exocytosis in these cells. Our conclusions are based on the following principal observations:

1. Elimination of synaptotagmin-7 alone impairs Ca$^{2+}$-triggered exocytosis, with an $\approx$50% decrease observed by capacitance measurements or amperometry in good agreement with recent data from insulin-secreting cells (31, 32).

2. Elimination of synaptotagmin-7 causes an apparent acceleration of the fast burst of exocytosis, probably by uncovering a faster, synaptotagmin-1-responsive subpool of the RRP.

3. The synaptotagmin-7 KO phenotype is essentially phenocopied when synaptotagmin-7 is unable to bind Ca$^{2+}$ by its C2B domain.

4. The double KO of both synaptotagmin-1 and -7 abolishes both the fast- and slow-burst components, leaving only a very slow, linear secretory component.

The apparent acceleration of the fast exocytotic burst in the synaptotagmin-7 KO appears to happen because of heterogeneity within the RRP (see above), such that the elimination of synaptotagmin-7 leaves a faster releasing subpool behind. This subpool might agree or overlap with the high Ca$^{2+}$-sensitive vesicle pool described by the Gillis laboratory (33). Consequently, during mild stimulation regimes (Figs. 1 and 3), the effect of the synaptotagmin-7 deletion is modest, which agrees well with the viability of the synaptotagmin-7 KO mice.

During stronger stimulus regimes, recruitment processes dominate exocytosis. The synaptotagmin-7 KO led to a $\approx$2-fold decrease in the sustained-release component, regardless of whether synaptotagmin-1 was present or not. The synaptotagmin-1 KO, in contrast, does not change the sustained component or the size of the slow exocytotic burst, representing fusion of slowly releasable vesicles. A model that can explain these findings is that synaptotagmin-7 engages the secretory machinery first, during vesicle priming, and that it is then partly or completely replaced by synaptotagmin-1 as the vesicle further matures and moves into the readily releasable pool characterized by a higher release rate. In this competition model, it would be expected that overexpression of synaptotagmin-1 leads to a speed-up in the apparent fusion kinetics of the burst, because of a larger fast component, which is exactly what we have found (8). The model is also consistent with the observations that the synaptotagmin-1 KO slows down the exocytotic burst and that the synaptotagmin-7 KO in the synaptotagmin-1 null background eliminates the residual slow burst.

Fig. 5. Synaptotagmin-1 and -7 both determine the secretory-burst components. Mean responses to flash stimulation in Syt-1/Syt-7 double-KO (dKO) cells (Syt 1 $\rightarrow$ KO, Syt 7 $\rightarrow$ KO, gray, n = 58, N = 6), control littermates (Syt 1 $\rightarrow$ KO, black, n = 33, N = 4), and Syt 1 KO (Syt 1 $\rightarrow$ KO, red trace, n = 26, N = 3) are shown; the residual exocytosis in synaptotagmin 1/7 dKO cells was almost devoid of a secretory burst and was linear in appearance.
depend almost entirely on synaptotagmin-7, but in the presence of synaptotagmin-1, part of the burst is clearly independent of synaptotagmin-7. This indicates that the slow burst in the presence and absence of synaptotagmin-1 has different origins, even though the amplitude and apparent fusion kinetics appears to stay constant. Thus, the slow burst is not a state attributable to a specific Ca^{2+} sensor but is, rather, a consequence of some vesicles being in one of several possible conditions that are suboptimal for fast release.

The elimination of burst-like exocytosis and reduction of overall exocytosis by >70% in the synaptotagmin-1 and -7 double-KO mice shows that exocytosis in chromaffin cells is similar to neuronal exocytosis in that it requires the presence of Ca^{2+}-binding synaptotagmin isoforms. These data demonstrate that synaptotagmins are strictly required for the release process itself and not just for synchronizing release that would happen anyway, as suggested (6). We demonstrate that at least in chromaffin cells, synaptotagmin-7 functions by binding to Ca^{2+} and, thus, in this sense, is a Ca^{2+} sensor. The finding that Ca^{2+} binding to the C2B domain of synaptotagmin-7 is essential is in agreement with similar findings for synaptotagmin-1 (7, 34–36). This similarity makes it likely that synaptotagmin-7 acts to trigger exocytosis in a manner similar to synaptotagmin-1, by binding to phospholipids and SNARE proteins (37, 38). Our data thus indicate that in chromaffin cells, synaptotagmin-1 and -7 are competing dual Ca^{2+}-sensors sharing a single common mechanism but with distinct kinetic properties.

Materials and Methods

Generation of Mouse Lines.
The generation and analysis of synaptotagmin-7 mutant mice has been described (27).

Preparation of Embryonic and Adult Mouse Chromaffin Cells.
Embryonic (E18) adrenal glands were dissected and chromaffin cells prepared by enzymatic (papain) digestion as explained (29). For adult adrenals, the following modifications were made: The glands were gently opened with tweezers before digestion, and, after trituration with a 1-ml pipette tip, the cell suspension was briefly centrifuged (4 min, 180 × g) and the pellet gently dispersed in culture medium. After plating, cells were incubated at 8% CO_{2}, 37°C, 95% relative humidity and used within 1–4 days. Details of the cell isolation technique are found in SI Materials and Methods.

Capacitance, Amperometry, and Intracellular Ca^{2+} Measurements.
Membrane capacitance whole-cell recordings were conducted essentially as described (17) by using an EPC-9 amplifier (HEKA Elektronik). Amperometric recordings were carried out by using 10-μm carbon fibers (P-100S; Amoco) insulated by electrodeposition insulating paint. Fibers cut at their tips were held in close contact with the cell and held at 800 mV versus the reference electrode. For depolarization experiments, the cells were filled with a solution containing 112 mM glutamate acid, 36 mM Hapes, 17 mM NaCl, 1 mM MgCl_{2}, 2 mM Mg-ATP, 0.3 mM GTP, 0.4 mM Fura-4F, 0.4 mM Fulupara 0.4 pH 7.2 with CsOH; osmolarity ~300 mOsm. Ca^{2+}-uncaging experiments, intracellular [Ca^{2+}]_i measurements by dual-dye ratiometric fluorimetry, and data analysis were performed as described (17, 39). The basal free-Ca^{2+} concentration was ~300–600 nM and adjusted if needed to the desired preflush level by photo-release small amounts of Ca^{2+} by brief monochromator illumination. All experiments were conducted at room temperature.

Statistical Analysis.
Significance levels of observed differences were assessed by using nonparametric Mann–Whitney test (*, P < 0.05, **, P < 0.01, ***, P < 0.001). Values are presented as mean values ± SEM.

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Figure 6
Schonn et al.