Synaptobrevin 1 mediates vesicle priming and evoked release in a subpopulation of hippocampal neurons

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Zimmermann J, Trimbuch T, Rosenmund C. Synaptobrevin 1 mediates vesicle priming and evoked release in a subpopulation of hippocampal neurons. J Neurophysiol 112: 1559–1565, 2014. First published June 18, 2014; doi:10.1152/jn.00340.2014.—The core machinery of synaptic vesicle fusion consists of three soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, the two t-SNAREs at the plasma membrane (SNAP-25, Syntaxin 1) and the vesicle-bound v-SNARE synaptobrevin 2 (VAMP2). Formation of the trans-oriented four-α-helix bundle between these SNAREs brings vesicle and plasma membrane in close proximity and prepares the vesicle for fusion. The t-SNAREs are thought to be necessary for vesicle fusion. Whether the v-SNAREs are required for fusion is still unclear, as substantial vesicle priming and spontaneous release activity remain in mammalian mass-cultured synaptobrevin/cellubrevin-deficient neurons. Using the autaptic culture system from synaptobrevin 2 knockout neurons of mouse hippocampus, we found that the majority of cells were devoid of any evoked or spontaneous release and had no measurable readily releasable pool. A small subpopulation of neurons, however, displayed release, and their release activity correlated with the presence and amount of v-SNARE synaptobrevin 1 expressed. Comparison of synaptobrevin 1 and 2 in rescue experiments demonstrates that synaptobrevin 1 can substitute for the other v-SNARE, but with a lower efficiency in neurotransmitter release probability. Release activity in synaptobrevin 2-deficient mass-cultured neurons was massively reduced by a knockdown of synaptobrevin 1, demonstrating that synaptobrevin 1 is responsible for the remaining release activity. These data support the hypothesis that both t- and v-SNAREs are absolutely required for vesicle priming and evoked release and that differential expression of SNARE paralogs can contribute to differential synaptic coding in the brain.

SNARE; neurotransmitter release; spontaneous release; release probability; short-term plasticity

RELEASE OF NEUROTTRANSMITTERS in the mammalian brain is mediated by fusion of synaptic vesicles (SVs) with the neuronal plasma membrane. This process has to be both fast and reliable and is performed by evolutionarily conserved protein machinery (Rizo and Rosenmund 2008; Sudhof 2013). Three proteins from the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) family are considered to bring the two membranes in close proximity and carry out membrane fusion (Sollner et al. 1993; Weber et al. 1998). A v-SNARE (anchored in the SV membrane) and the t-SNAREs syntaxin (Bennett et al. 1992) and synaptosomal-associated protein 25 (SNAP-25) (Oyler et al. 1989) (both anchored in the plasma membrane) zipper up from their NH2 to COOH terminus and thus form a four-α-helix bundle called the trans-SNARE complex (Poirier et al. 1998; Sorensen et al. 2006; Sutton et al. 1998). Whether fusion of SVs is possible without SNARE proteins is still an open question. The major v-SNARE paralog on SVs in the mammalian brain is synaptobrevin 2 (syb2), also known as vesicle-associated membrane protein 2 (VAMP2) (Elferink et al. 1989). Loss of the syb2 homolog in Drosophila abolishes evoked release entirely and reduces spontaneous release significantly (Deitcker et al. 1998). Elimination of the two v-SNARE isomers present in Caenorhabditis elegans leads to severe defects in synaptic transmission (Nonet et al. 1998). In mouse the syb2 knockout (KO) has been studied in high-density cultures of hippocampal neurons (Schoch et al. 2001). Even though Ca2+-mediated evoked release is virtually absent without syb2, the pool of readily releasable vesicles (RRP) is only reduced to ~10% of wild-type (WT) levels and spontaneous fusion of SVs can still be observed. The additional depletion of cellubrevin (VAMP3) in these cells revealed no aggravated phenotype, which speaks against a compensatory effect of this v-SNARE in the syb2 KO background (Deak et al. 2006). In this study we addressed the question of how syb2-deficient hippocampal neurons can still promote fusion. We used the autaptic culture system (Bekkers and Stevens 1991) to study release from individual cells rather than sampling input from several neurons as done in mass culture. We characterized synaptobrevin 1 (syb1, VAMP1) as a v-SNARE in SV exocytosis by a molecular rescue approach and found that this paralog of syb2 is expressed in a subset of hippocampal neurons in our syb2 KO culture. We could correlate the size of the RRP of individual neurons to their syb1 expression levels and showed that knocking down syb1 dramatically decreases the RRP of mass-cultured syb2 KO neurons.

MATERIALS AND METHODS

Neuronal culture. All procedures for experiments involving animals were approved by the animal welfare committee of Charité Medical University and the Berlin state government Agency for Health and Social Services (license no. T 0220/09). Male and female mouse embryos [18.5 days old (E18.5)] were delivered by cesarean section. Hippocampal neurons from syb2 KOs (Schoch et al. 2001) and WT littermates were dissected out and plated on astrocyte feeder islands as described previously (Xue et al. 2007). Electrophysiological recordings were performed after 9–16 days in vitro (DIV).

Lentiviral constructs and virus production. Sequences of murine syb1 and syb2, respectively, were cloned into a lentiviral shuttle vector under the control of a human synapsin-I promoter. To enable identification of infected cells the expression cassette of synaptobrevin was fused to a nuclear localization sequence-tagged red fluorescent protein (NLS-RFP) via a self-cleaving P2A peptide (Kim et al. 2011). For syb1 knockdown a murine syb1-specific small interfering RNA (siRNA) target sequence (5’-CAG GCC GGT ATT GCA GAC C-3’) was obtained with Genscript siRNA Target Finder (https://www.genscript.com/siRNA/app/rnai) and cloned as short hairpin
RNA (shRNA) into a lentiviral shuttle vector under the control of a U6 promoter. To identify infected neurons, the shuttle vector contained a human synapsin-I promoter, which drives the expression of a NLS-RFP. For knockdown experiments neurons were transduced either with the syb1 shRNA construct or with a scrambled control.

Lentiviral particles were prepared as described by Lois et al. (2002). For transduction, ~1 x 10⁶ infectious particles per 35-mm diameter well were pipetted onto 1 DIV hippocampal neurons.

**Electrophysiology.** Whole cell patch-clamp recordings were performed in a standard extracellular solution containing the following (in mM): 140 NaCl, 2.4 KCl, 10 HEPES (Merck, Darmstadt, Germany), 10 glucose (Carl Roth, Karlsruhe, Germany), 2 CaCl₂ (Sigma-Aldrich, St. Louis, MO), and 4 MgCl₂ (Carl Roth); 300 mosM, pH 7.4. To block glutamatergic or GABAergic responses, 10 μM 2,3-dioxy-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Tocris Bioscience, Bristol, UK) and 30 μM bicineulline (Tocris Bioscience), respectively, were added to the extracellular solution. In mass culture experiments voltage-gated sodium channels were blocked by addition of 0.5 μM tetrodotoxin (TTX) (Tocris Bioscience). To assess the size of the RRP, the extracellular solution was made hypertonic by adding 500 mM sucrose (Sigma-Aldrich) (described in Rosenmund and Stevens 1996). Internal solution contained the following (in mM): 136 KCl, 17.8 HEPES, 1 EGTA (Carl Roth), 4.6 MgCl₂, 4 Na₂ATP, 0.3 Na₃GTP (Sigma-Aldrich), and 12 creatine phosphate (Calbiochem, Darmstadt, Germany), with 50 U/ml phosphocreatine kinase (Sigma-Aldrich); 300 mosM, pH 7.4. In some experiments, 5 mM fixable Cascade Blue (Life Technologies, Darmstadt, Germany) was added to the internal solution. Borosilicate glass pipettes (Science Products, Hofheim, Germany) had a resistance of 2–3.5 MΩ. All recordings were performed with a Multiclamp 700B amplifier under control of Clampex 10.0 (both Molecular Devices, Sunnyvale, CA). Data were acquired at 10 kHz and filtered at 3 kHz.

Excitatory (EPSC) and inhibitory (IPSC) postsynaptic currents were recorded after a 2-ms depolarization from ~70 mV to 0 mV. The vesicular release probability was calculated by dividing the charge of the EPSC by the charge of the RRP. The 10 Hz protocol consisted of 50 depolarization stimuli within 5 s, and the responses were normalized to the first EPSC.

For background noise subtraction, miniature postsynaptic currents were recorded in the presence and absence of 10 μM NBQX and 30 μM bicineulline, respectively. Analysis was performed after filtering data at 1 kHz and then running a template-based detection algorithm implemented in A xoGraph X 1.5.4 (A xoGraph, Berkeley, CA).

**Immunocytochemistry.** Cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) for 10 min after 14 DIV or directly after transfection. Primary antibodies were purchased from Synaptic Systems (Göttingen, Germany) (anti-syb2, catalog no. 104211; anti-synaptophysin 1, catalog no. 101004) and Abcam (Cambridge, UK) (anti-syb1, catalog no. ab3346). Cells were incubated with primary antibodies at 4°C overnight. Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Images were acquired on an Olympus (Tokyo, Japan) IX81 epifluorescent microscope with a UPLSAPO ×60 water immersion objective and MetaMorph software (Molecular Devices). To correlate electrophysiological data with immunolabeling we used Imaris software (Digital Imaging). Images were acquired on an Olympus Fluoview (Tokyo, Japan) confocal microscope with a 60× water immersion objective. To calculate the size of the RRP, using pulsed application of hypertonic solution (Rosenmund and Stevens 1996). Vesicles that are fusion competent are forced to fuse within 3–4 s after the neuron is exposed to external solution containing an additional 500 mM sucrose. Surprisingly, we found that 47 of 54 cells lacking syb2 exhibited no measurable RRP, and again all but one WT cell had robust responses (Fig. 1, C and D). The pool size of those seven KO cells responding to sucrose was 56 ± 35 pc on average compared with 366 ± 50 pc in WT (n = 55). Only one KO neuron had a WT-sized pool (see cell 1 in Fig. 2A). The lack of readily releasable vesicles in most syb2 KO neurons was also evident when we examined spontaneous fusion events (“minis”) (Fig. 1E). Only 3 of 47 cells in KO showed detectable miniature EPSCs (mEPSCs) compared with 46 of 48 in WT (Fig. 1F). The average amplitude of detected minis in KO and WT was 12.5 ± 1.7 pA and 24.7 ± 1.0 pA, respectively (Fig. 1G). It should be mentioned that we also found two inhibitory neurons in our syb2 KO culture with considerable evoked current responses (6.8 and 0.9 nA) and pool sizes (3.4 and 1.8 nC). Because of the low numbers of GABAergic neurons in our culture we did not perform any further statistical analysis on those cells.

**Results.** A subpopulation of neurons exhibits release in syb2 KO autaptic culture. To study the nature of release activity in the absence of syb2 we grew murine hippocampal neurons from syb2 KO (Schoch et al. 2001) and WT littermates on glial microarrays and performed whole cell patch-clamp experiments on autaptic neurons. Consistent with previous findings (Guzman et al., 2010), excitatory evoked release was virtually absent in the KO group (Fig. 1). While the WT control group showed robust evoked glutamatergic current responses (3.4 ± 0.5 nA (SE)) in 54 of 55 neurons, we detected a single cell out of 59 KO neurons that displayed a quantifiable evoked response (0.64 nA) (Fig. 1, A and B). We next measured the RRP, using pulsed application of hypertonic solution (Rosenmund and Stevens 1996). Vesicles that are fusion competent are forced to fuse within 3–4 s after the neuron is exposed to external solution containing an additional 500 mM sucrose. Surprisingly, we found that 47 of 54 cells lacking syb2 exhibited no measurable RRP, and again all but one WT cell had robust responses (Fig. 1, C and D). The pool size of those seven KO cells responding to sucrose was 56 ± 35 pc on average compared with 366 ± 50 pc in WT (n = 55). Only one KO neuron had a WT-sized pool (see cell 1 in Fig. 2A). The lack of readily releasable vesicles in most syb2 KO neurons was also evident when we examined spontaneous fusion events (“minis”) (Fig. 1E). Only 3 of 47 cells in KO showed detectable miniature EPSCs (mEPSCs) compared with 46 of 48 in WT (Fig. 1F). The average amplitude of detected minis in KO and WT was 12.5 ± 1.7 pA and 24.7 ± 1.0 pA, respectively (Fig. 1G). It should be mentioned that we also found two inhibitory neurons in our syb2 KO culture with considerable evoked current responses (6.8 and 0.9 nA) and pool sizes (3.4 and 1.8 nC). Because of the low numbers of GABAergic neurons in our culture we did not perform any further statistical analysis on those cells.

**Pool size in syb2 KO neurons correlates to syb1 expression levels.** An earlier study in syb2 KO high-density culture (Schoch et al. 2001) reported an overall ~90% decrease in RRP size and mEPSC frequency in excitatory neurons. However, in the experimental conditions of high-density neuronal culture, postsynaptic neurons sample input from several presynaptic neurons. In syb2 KO autaptic culture, where each neuron receives input only from itself, we found that most of the cells are incapable of releasing any neurotransmitter and...
only a small subpopulation showed release (Fig. 1). This result may be explained by the presence of another paralog of syb2 in the neurotransmitter-releasing subpopulation, such as syb1. While the initial study had no evidence for syb1 expression (Schoch et al. 2001), we reevaluated the putative role of this paralog by correlating syb1 expression with the ability of individual cells to release competent. To examine whether syb1 may be responsible for vesicle priming in the absence of syb2, we correlated RRP size with syb1 protein levels. Individual syb2 KO neurons were recorded and labeled with a fixable fluorescent dye present in the patch pipette solution. After recording, the neurons were fixed and stained with a syb1 antibody (Fig. 2A). We identified the recorded cells and plotted the pool size against the levels of syb1 protein (normalized to synaptophysin 1 levels; see MATERIALS AND METHODS). This analysis exhibited a strong correlation between syb1 levels and RRP size (r = 0.91) (Fig. 2B), indicating that syb1 may be responsible for remaining release in syb2 KO neurons.

Release properties differ between syb1 and syb2. To provide evidence that syb1 expression is capable of mediating vesicle priming, we performed syb1 rescue experiments in syb2-deficient neurons. We expressed syb1 in syb2 KO autaptic neurons, using a lentiviral expression system (Lois et al. 2002). We compared the electrophysiological parameters to WT as well as syb2 KO neurons in which we expressed syb2 with the same shuttle vector as syb1. Both syb paralogs robustly rescued the deficiency in evoking synaptic responses (Fig. 3). We noted, however, that the EPSC amplitude size for syb1 rescues was significantly reduced (2.2 ± 0.3 nA) compared with the syb2 rescues (3.6 ± 0.6 nA) (Fig. 3, A and B). The reduction in EPSC size was not due to a smaller RRP as determined by pulsed sucrose application (Fig. 3, C and D). Instead, we observed a decreased vesicular release probability in the syb1 rescued compared with syb2 and WT (Fig. 3E). This was confirmed by two additional experiments. First, comparative analysis of response amplitudes during repetitive 10-Hz stimulation experiments showed that syb1-rescued neurons displayed less depression than the syb2 rescue group (Fig. 3F), a hallmark for reduced release probability (Zucker and Regehr 2002). Second, the frequency of spontaneous release was significantly reduced in the syb1 rescue compared with syb2 rescue and WT (Fig. 3, G and H), indicating a higher energy barrier for fusion-competent vesicles. mEPSC amplitudes were unaltered [syb1 rescue: 20.5 ± 1.5 pA (n = 43); syb2 rescue: 21.0 ± 1.0 pA (n = 56); WT: 21.8 ± 1.3 pA (n = 28)]. While it is possible that the change in release probability is caused by a change in relative expression levels of the two synaptobrevin paralogs, we consider it unlikely, as both constructs rescue vesicle priming equally well. Furthermore, synaptic function in our system appears relatively insensitive to changes in expression levels, as syb2 heterozygous (het) deficient neurons do not display significant changes in vesicle release, priming, or release probability (Pvr) (EPSC amplitudes: WT 6.4 ± 1.2 nA, syb2 het 6.1 ± 1.1 nA; RRP size: WT 1.0 ± 0.2 nC, syb2 het 0.9 ± 0.2 nC; Pvr: WT 6.0 ± 0.7%, syb2 het 5.6 ± 0.6%; n = 22 each from 2 cultures). Taken together, the rescue experiments demonstrate that syb1 can substitute for loss of syb2 in terms of maintenance of neurotransmitter release in central synapses. However, syb1 promotes evoked and spontaneous release less efficiently compared with syb2.
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syb1 promotes release in syb2 KO mass culture. To test whether syb1 is responsible for the remaining release observed in syb2 KO mass cultures (Schoch et al. 2001), we genetically manipulated syb1 expression in syb2 KO cultures using shRNA knockdown. Western blot analysis showed that syb1 is indeed expressed in mass-cultured hippocampal neurons and that the shRNA successfully suppresses the expression of syb1 (Fig. 4A). We subsequently assessed the size of the input RRP in three groups: WT, syb2 KO, and syb2 KO+syb1 knockdown (KD) neurons. Since in mass cultures the input to the recorded cell can be both glutamatergic and GABAergic, we added either bicineulline or NBQX to our bath solutions to determine the impact of this manipulation on both neurotransmitter systems. We confirmed the reduction of RRP size in syb2 KO compared with WT (Fig. 4, B and C). The syb2 KO+syb1 KD group showed an additional reduction of pool size to 0.7 ± 0.2% of WT levels in the presence of bicuculline and 0.3 ± 0.1% of WT levels in the presence of NBQX (Fig. 4C). These findings indicate that syb1 is an important v-SNARE for rendering vesicles fusion competent in the syb2 KO. Surprisingly, we detected only a slight further reduction in the frequency of mEPSCs and no change in mIPSC frequency in the syb1 knockdown (Fig. 4D).

**DISCUSSION**

SNAREs are considered to be essential components of the SV fusion process, yet whether v-SNAREs are absolutely required for vesicle fusion is currently unclear. In this study we used the advantages of the autaptic cell culture system to examine the loss of syb2, the major v-SNARE in hippocampal neurons. Our major findings are as follows: 1) The majority of syb2-deficient neurons, when grown on isolated microislands, are devoid of any Ca^{2+}-mediated evoked or spontaneous release and have no measurable RRP (Fig. 1). In a small subpopulation of neurons, however, we detected the expression of the v-SNARE paralog syb1 and found a correlation between syb1 protein levels and remaining RRP size (Fig. 2). This supports the hypothesis that v-SNAREs are absolutely required for vesicle fusion. 2) In rescue experiments with syb1 and syb2 we observed that syb1 is less efficient in release, which is reflected in reduced EPSC amplitudes and a decreased release probability and mEPSC frequency (Fig. 3).

We interpret the remaining responses in mass-cultured syb2 KO neurons (Deak et al. 2004, 2006; Schoch et al. 2001) to be caused by a subpopulation of neurons that express syb1 (Fig. 4). Our results clearly argue that v-SNAREs are mandatory for evoked release and the fusion competence of synaptic vesicles.

To our knowledge, syb1 has not yet been characterized in the fusion of SVs at central synapses. Whereas syb2 is the dominant v-SNARE with a high release probability, syb1 could promote fusion in cells that depend on lower release probability and less efficient release. The usage of different v-SNARE paralogs on SVs might thus serve as a vesicle-intrinsic determinant of release probability. Deak et al. (2004) also examined syb2 KO neurons in mass culture and found reduced release probability in those cells compared with WT neurons. Even though the authors did not attribute these findings to residual syb1 levels at that time, they fit very well with our results that syb1 possesses an intrinsically lower release probability compared with syb2. Carrying out styryl dye and HRP uptake experiments, Deak et al. (2004) studied exo- and endocytosis in syb2 KO neurons. Interestingly, the authors did not report any obvious variance between individual synapses, as would be expected if syb1 mediates release in a subpopulation of cells. The discrepancy between these uptake studies and our electrophysiological analyses remains to be studied.

The mouse neuromuscular junction (NMJ) has already been shown to depend on syb1 in Ca^{2+}-triggered exocytosis (Liu et al. 2011). The authors found a reduction in spontaneous and evoked neurotransmitter release as well as lower initial vesicular release probability in syb1 KO NMJs compared with WT. In contrast to our findings, they did not find a decrease in pool size when syb1 levels were reduced. Instead, they report a reduced sensitivity and cooperativity to calcium in the KO neurons. However, because of the relatively high expression of syb2 in the NMJ it is rather difficult to directly compare the two studies, as the number of syb proteins present is supposedly very different in the two systems. We find it remarkable, though, that the mouse NMJ, consisting of thousands of unreliable single vesicle release sites (Meriney and Dittrich 2013), depends at least

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**Fig. 2.** RRP size is correlated to synaptobrevin 1 (syb1) expression levels in syb2-deficient neurons. A: RRP traces of 2 exemplary syb2 KO autaptic neurons that have been filled with Cascade Blue dye during recordings (left) and their corresponding post hoc immunostainings for syb1 (right). Evoked response of cell 1 is shown in Fig. 1A. Scale bars, 20 μm. B: plot of RRP size against the intensity of syb1 fluorescence normalized to the fluorescence intensity of synaptic marker synaptophysin 1 (syb1). Cells 1 and 2 shown in A are indicated in red and gray, respectively. syb1 OE, mean RRP size and syb1 expression levels of the syb1 rescue (Fig. 3D) (error bars show SE). Pearson’s correlation coefficient $r = 0.91$, $P < 0.001$ (data point for syb1 OE was excluded from the correlation analysis; see MATERIALS AND METHODS).
partially on syb1—a v-SNARE that we show to mediate release with lower release probability.

Interestingly, rescue studies with cellubrevin (VAMP3), also closely related to syb2, reported a complete compensation of the loss of syb2 in neurons (Deak et al. 2006) and chromaffin cells (Borisovska et al. 2005). The sequence homology between syb1 and syb2 is 76%, with the largest differences in the NH₂ terminus. However, cellubrevin, which can completely compensate the loss of syb2, does not possess a sizable NH₂ terminus beyond the SNARE motif and shares only 74%
sequence homology with syb2. So it remains to be studied which part of the sequence is responsible for the reduced release efficiency of syb1.

Our finding that syb1 has a significantly lower release probability compared with syb2 might explain why the majority of syb2 KO neurons in our autaptic culture that still had a measurable RRP did not show evoked responses. Studies from syntaxin hypomorphs have shown that a reduction in the intrinsically lower release probability of syb1 would even further reduce the likelihood of Ca\textsuperscript{2+}-evoked fusion, causing it to drop below detection levels.

Whereas RRP size in the syb2 KO can be correlated to syb1 expression, the knockdown of syb1 did not affect miniature events to the same extent. There are two different possible explanations: The mini frequency in syb2 KO mass culture is already quite low [0.6–1 Hz (compare also Deak et al. 2006; Schoch et al. 2001)]. It is conceivable that spontaneous fusion at this low rate can still be maintained with the reduced RRP that we saw in the syb1 knockdown. Alternatively, spontaneously fusing vesicles may originate from a different pool than the RRP (Ramirez et al. 2012; Sara et al. 2005). In the scenario in which syb2 is absent, syb1 drives fusion of the RRP whereas an additional v-SNARE may exclusively drive spontaneous fusion. A possible candidate could be the noncanonical SNARE Vps10p-tail-interactor-1a (vti1a), which has been shown to promote spontaneous release in the absence of syb2 (Ramirez et al. 2012). Similarly, in Drosophila Ca\textsuperscript{2+}-evoked release is thought to be driven by the v-SNARE n-syb, whereas spontaneous release persists even in the absence of n-syb (Deitcher et al. 1998; Yoshihara et al. 1999). Studying a complete genetic deletion of syb1 and syb2 could help to differentiate between the two explanations.

Taken together, our results support the hypothesis that v-SNAREs are absolutely required for evoked release and vesicle priming at central synapses. Furthermore, differential expression of SNARE paralogs can contribute to making vesicle release probability and short-term plasticity characteristics more diverse among different synapses.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: J.Z. and C.R. conception and design of research; J.Z. and T.T. performed experiments; J.Z. and T.T. analyzed data; J.Z. and C.R.
interpreted results of experiments; J.Z. prepared figures; J.Z. drafted manuscript; T.T. and C.R. edited and revised manuscript; J.Z., T.T., and C.R. approved final version of manuscript.

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