Adiponectin regulates contextual fear extinction and intrinsic excitability of dentate gyrus granule neurons through AdipoR2 receptors

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating condition that is characterized by intense feelings of fear and severe flashbacks that follow a traumatic experience. The development of PTSD symptoms could result from the inability to use contextual information to modulate fear expression and extinction.1-4 The hippocampus is a key brain region for the context specificity of fear and extinction memories.3-9 Hippocampal lesions selectively impair fear responses to the context.7,10 Volumetric reductions in the hippocampus, in particular the dentate gyrus (DG), have been reported to be associated with PTSD.11-15 The DG serves as a gateway for information flow through the hippocampal formation16 and has an essential role in generating contextual fear memories.3,17-21 Reactivation of DG neurons activated during fear conditioning is sufficient to induce freezing behavior.21,22 However, the exact regulatory mechanisms through which the DG influences context-specific fear responses are poorly understood.

The hippocampus is well positioned to sense and respond to changes in peripheral hormonal signals, as it is lying alongside the choroid plexus and adjacent to the cerebral ventricles. Adiponectin is a circulating hormone that is secreted almost exclusively by adipocytes.23,24 In the plasma, adiponectin exists as a full-length and a globular form generated by the cleavage of full-length adiponectin. Adiponectin aggregates in multimeric complexes including trimers, hexamers and high-molecular-weight forms.25,26 It is known that adiponectin can cross the blood–brain barrier from the systemic circulation. Adiponectin can be detected in the cerebrospinal fluid after an intravenous injection in adiponectin knockout mice.27,28 Low- molecular-weight forms of adiponectin are found in the cerebrospinal fluid of humans and mice.27,29-31 The biological effects of adiponectin are mediated mainly through two receptor subtypes, AdipoR1 and AdipoR2, with 67% amino acid identity.32 These receptors contain 7-transmembrane domains such as G-protein-coupled receptors but they are opposite to the membrane topology of all known G-protein-coupled receptors, with their intracellular N terminus and extracellular C terminus.33 AdipoR1 and AdipoR2 have distinct distribution patterns in various tissues and different binding affinity for globular and full-length adiponectin.32 Despite ample evidence for the roles of AdipoR1 and AdipoR2 in metabolic regulation, there are scarce data concerning their involvement in higher brain function. Both AdipoR1 and AdipoR2 are highly expressed in the DG, suggesting that this brain region is a major target of adiponectin action in the brain. However, whether adiponectin and its receptors in this brain region contribute to contextual control of fear expression and extinction has not been extensively explored in PTSD.

Post-traumatic stress disorder (PTSD) is characterized by exaggerated fear expression and impaired fear extinction. The underlying molecular and cellular mechanisms of PTSD are largely unknown. The current pharmacological and non-pharmacological treatments for PTSD are either ineffective or temporary with high relapse rates. Here we report that adiponectin-deficient mice exhibited normal contextual fear conditioning but displayed slower extinction learning. Infusions of adiponectin into the dentate gyrus (DG) of the hippocampus in fear-conditioned mice facilitated extinction of contextual fear. Whole-cell patch-clamp recordings in brain slices revealed that intrinsic excitability of DG granule neurons was enhanced by adiponectin deficiency and suppressed after treatment with the adiponectin mimetic AdipoRon, which were associated with increased input resistance and hyperpolarized resting membrane potential, respectively. Moreover, deletion of AdipoR2, but not AdipoR1 in the DG, resulted in augmented fear expression and reduced extinction, accompanied by intrinsic hyperexcitability of DG granule neurons. Adiponectin and Adiponectin failed to induce facilitation of fear extinction and elicited inhibition of intrinsic excitability of DG neurons in AdipoR2 knockout mice. These results indicated that adiponectin action via AdipoR2 was both necessary and sufficient for extinction of contextual fear and intrinsic excitability of DG granule neurons, implying that enhancing or dampening DG neuronal excitability may cause resistance to or facilitation of extinction. Therefore, our findings provide a functional link between adiponectin/AdipoR2 activation, DG neuronal excitability and contextual fear extinction, and suggest that targeting adiponectin/AdipoR2 may be used to strengthen extinction-based exposure therapies for PTSD.

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investigated so far. Here we set out to identify the role of adiponectin by using mice deficient in adiponectin or received intra-DG infusions of adiponectin, to test them in a Pavlovian contextual fear-conditioning and extinction paradigm. This paradigm has been used as a model of fear acquisition relevant to some aspects of PTSD and mimicking extinction-based exposure therapies.34-36 The involvement of AdipoR1 and AdipoR2 in contextual fear memories was further investigated using specific receptor knockout mice. In order to understand the cellular mechanisms underlying the actions of adiponectin and its receptors, we also examined the intrinsic excitability of DG granule neurons using whole-cell patch-clamp recordings.

MATERIALS AND METHODS

Animals

Adult wild-type (WT) male and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in groups of five under a 12-h light/dark cycle (lights on at 0900 h) with ad libitum access to food and water. All procedures were carried out in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committees of University of Texas Health Science Center at San Antonio.

AdipoR1/<-/- mice. Heterozygous adiponectin gene knockout mice on a C57BL/6J genetic background were generated as previously described and intercrossed to generate adiponectin knockout (AdipoR1<->) mice and WT littermates.37 Genotyping was performed by screening for the adiponectin null allele as described previously.37

AdipoR2<-> mice. Heterozygous AdipoR2 gene knockout mice were obtained from Deltagen (San Carlos, CA, USA), which had been backcrossed at least six generations to C57BL/6J mice by the Jackson Laboratory. AdipoR2<-> mice were intercrossed to produce AdipoR2<-> mice and WT littermates. Screening for the AdipoR2-null allele was performed as described previously.37

Generation of AdipoR1<->lox<-> mice (AdipoR1<->lox<->lox). A targeting vector was designed in which exon 2 of the AdipoR1 gene was flanked by loxP sites. Exon/intron boundary analysis revealed that the deletion of this exon led to a frame shift and the generation of a null mutant of AdipoR1. A 11.5-kb genomic clone derived from C57BL/6J mice containing exons 2–5 of the AdipoR1 gene was obtained and used to generate the AdipoR1 loxP targeting vector (Figure 3a). The targeting construct was generated by inserting a loxP site, a sequence containing a PGK-neo expression unit and the HI site located 232 bp upstream of exon 2 and two Frt sites into the BamHI site located 232 bp upstream of exon 2 and the other loxP site into the SacI site 295 bp downstream of exon 2. The targeting construct contains 5.3 kb of homologous DNA upstream of the first loxP site and 3.3 kb of homologous DNA downstream of the second loxP site. Bruce4 embryonic stem (ES) cells derived from C57BL/6J mice were microinjected with the targeting vector. Positive cells were selected and screened for homologous recombination and incorporation of the IoxP sites. Homologous recombination was confirmed with PCR to detect the WT and AdipoR1<->lox<-> alleles using the primers forward-5′-CTGGGGATAGTTCTGGAT-3′ and reverse-5′-TTACTACTGGGCTCTTGGT-3′, to detect the downstream loxP site and was confirmed using Southern blot analysis. ES cell clones that were found to be AdipoR1<->lox<-> were injected into B6.Cg-Tg(CAG-tdTomato)2D9Rj/J-derivated blastocysts for generation of chimeras. Chimera were bred with albino B6.Cg-Tg(CAG-tdTomato)2D9Rj/J mice to obtain germine transmission. The mice with the floxed AdipoR1 allele were confirmed using Southern blot analysis (Figure 3a). These mice were intercrossed to generate AdipoR1<->lox<->lox mice. Removal of the neomycin selective marker flanked by Frt sites was accomplished by crossing the AdipoR1<->lox<->lox mice with a FLPe-deleter strain that expresses FLP recombinase (B6.129S4-Gt(RosA)26Ser8tm1(FLP14)Tim/J). AdipoR1<->lox<->lox mice used in this study were backcrossed to C57BL/6J for more than ten generations.

Generation of AdipoR1<->fl<->fl mice. AdipoR1<->fl<->fl mice were crossed with mice expressing Cre recombinase under the control of the proopio-melanocortin promoter (POMC-Cre),38 to generate AdipoR1<->lox<->lox POMC-Cre mice. POMC-Cre mice were bred with the Ai14-TdTomato reporter mice to confirm the Cre-mediated excision in the DG. Subsequently, male POMC-Cre/+ mice were crossed to female AdipoR1<->lox<->lox mice, to yield AdipoR1<->lox<->lox POMC-Cre/+ (termed AdipoR1<->POMC-Cre) mice and their offspring were used in the study. The PCR primers used for genotyping were as follows: POMC-Cre, forward-5′-GGGTTGCTGGCAGTAA AACTAC5′-3′, reverse-5′-GGGACACGACATGCTGTC5′-3′ and AdipoR1 WT and fllox, forward-5′-CTCTGGGGGATAGTTCTGGT-3′, reverse-5′-TTACTACAC TGGCCCTGCTGTG-3′.

RNA extraction and reverse transcriptase-PCR. Mice were decapitated and their brains were removed immediately and placed in a Petri dish containing pre-chilled phosphate-buffered saline. The DG was isolated under a stereomicroscope using a dissection procedure, as previously described.39 The isolated DG was homogenized and total RNA was extracted with PureLink RNA Mini Kit (Life Technologies). The primers used to amplify each product are as follows: AdipoR1 exon 2, forward-5′-CCCTGGATACACAGACACC5′-3′, reverse-5′-GGCAATGGGCTC TTCTGGT-3′ and 18s, forward-5′-CCGAGCACAGGATGACAG5′-3′, reverse-5′-GA TTCTGCCTACACCATG5′-3′.

Adult (8–12 weeks old) male mice of different genotypes were used for all behavioral and electrophysiological experiments.

Drugs

Recombinant mouse adiponectin (R&D Systems, Minneapolis, MN, USA) was dissolved in artificial cerebrospinal fluid (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl2·6H2O, 0.9 mM CaCl2·2H2O, 1.5 mM KH2PO4 and 8.1 mM NaHPO4). AdipoRon, 2-(4-benzoylphenoxy)-N-[1-(phenylmethyl)-4-piperidinyl]acetamide (AdipoGer, San Diego, CA, USA), was dissolved in dimethyl sulfoxide.

Behavioral tests

Contextual fear conditioning and extinction. Fear conditioning was performed in a mouse fear-conditioning chamber equipped with the Coulbourn Freezerframe system and positioned inside a sound-attenuating cabinet (Coulbourn Instruments, Whitehall, PA, USA). The rear wall of the cabinet was covered with black and white vertical striped vinyl wallpaper. The chamber consisted of 16 stainless steel rods (5-mm diameter), which were evenly spaced 1-cm apart (center-to-center) and connected to a shock generator and a scrambler for the delivery of foot shock. A houselight was located on one side wall of the chamber and served to illuminate the chamber. A digital camera was mounted on the top of the chamber for monitoring and recording animal behaviors. A stainless steel pan was placed beneath the grid floor and contained a sheet of paper sprayed with 20% isopropyl alcohol. Mice were trained in a contextual fear-conditioning paradigm.38 Mice were first acclimated to the conditioning chamber for 3 min. Next, a shock (0.8 mA, 2 s duration) was delivered four times with a 60-s inter-shock interval. Mice were removed from the conditioning chamber 60 s following the last shock. During extinction training, mice were repeatedly re-exposed to the conditioning chamber for 5 min without shock delivery once daily for 4 or 6 days. Freezing responses during the pre-conditioning phase, conditioning phase, extinction and retrieval testing were recorded and measured as an index of fear memory with the Freezeframe 3 software. The chamber was cleaned and dried using 20% isopropyl alcohol between each trial.

Elevated plus maze test. The maze consisted of two white acrylic open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm) that form the shape of a ‘+’ sign. The apparatus was elevated to a height of 70 cm from the floor. Mice were placed in the central square (5 × 5 cm) facing the corner between a closed arm and an open arm, and allowed to explore the elevated plus maze for 5 min. The percentage of open arm entries and the percentage of time spent in the open arms were quantified as a measure of anxiety. Total arm entries were analyzed as an index of exploratory activity.

Hot-plate test. Pain sensitivity was assessed by testing reflexes in response to a thermal stimulus. The surface of the hot plate was heated to a constant temperature of 55 °C. Mice were placed on the hot plate for a maximum duration of 90 s. The latency to respond with hind paw lick or
jump was recorded. After the mouse responded, it was immediately removed from the hot plate and returned to its home cage. If the mouse did not respond, the test was terminated at the end of 90 s.

**Visual cliff test.** A clear acrylic box with half of the inner surface covered with checkerboard paper was used for the mouse visual cliff test. The box was placed ~70 cm above the floor. A sheet of clear Plexiglas provided a solid horizontal surface in spite of the visual appearance of a cliff. Each mouse was placed at the midline of the box and the direction in which the mouse stepped was recorded on 10 consecutive trials.

**Stereotaxic surgery.**

- **Stereotaxic surgery was performed as described.**
- **Mice were anesthetized with an intramuscular injection of a cocktail containing ketamine (60 mg ml$^{-1}$), acepromazine (1 mg ml$^{-1}$) and xylazine (8 mg ml$^{-1}$) (0.1 ml kg$^{-1}$, intramuscular) and mounted onto a stereotaxic frame.**
- **For intra-DG microinjections, WT mice received bilateral guide cannula (23-gauge; Plastics One, Roanoke, VA, USA) targeting the DG (coordinates: anteroposterior (AP) = −2.1 mm, mediolateral (ML) = ±1.5 mm, dorsoventral (DV) = −1.2 mm from the bregma) as previously described.**
- **Following surgery, mice were individually housed, handled daily and allowed to recover for 7 days.**
- **Intra-DG microinjections were performed on conscious, unrestrained and freely moving mice in their home cage.**
- **On the experimental day, a 33-G stainless-steel injector connected to a 5-μl syringe was inserted into the guide cannula and extended 1 mm beyond the tip. Adiponectin (0.5 μg ml$^{-1}$) or artificial cerebrospinal fluid was infused bilaterally in a volume of 0.5 μl (0.25 μl per side) over 2 min. The injector tips were held in place for additional 5 min after the end of infusion to avoid backflow.**

**Whole-cell patch-clamp recordings**

- **Whole-cell patch-clamp recordings were conducted with the following conditions:**
  - 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl$_2$, 1 mM NaH$_2$PO$_4$, 11 mM glucose and 24 mM NaHCO$_3$.
  - 124 mM NaCl, 2 mM KCl, 2 mM MgSO$_4$, 1.25 mM NaH$_2$PO$_4$, 2 mM CaCl$_2$, 26 mM NaHCO$_3$, 10 mM d-glucose and 0.4 mM vitamin C.
- **Brain slices were constantly perfused with the extracellular bath solution during whole-cell patch-clamp recordings.**
- **Whole-cell recording pipettes with tip resistances between 4 and 7 MΩ were pulled from thin-walled borosilicate glass capillaries (TW-150 F, World Precision Instruments, Sarasota, CA, USA). Pipettes were filled with a potassium gluconate-based internal solution containing 120 mM potassium gluconate, 20 mM KCl, 2 mM MgCl$_2$, 10 mM HEPES, 2 mM ATP, 0.25 mM GTP and 0.1 mM EGTA, with pH adjusted to 7.4 and osmolality of 295 mOsm.

**RESULTS**

**Normal acquisition of contextual fear but impaired extinction learning in adiponectin knockout mice**

- **Adiponectin knockout (Adipo−/−) mice and WT littermates were trained to associate an unconditioned stimulus (4 foot shocks; 0.8 mA, 2 s duration, 60 s inter-shock interval) with a context (the shock chamber).**
- **During contextual fear-conditioning training, there was no difference between genotypes in the level of postshock freezing (Figure 1a), suggesting that adiponectin is not required for the acquisition of contextual fear.**
- **At 24 h after conditioning, extinction training was performed on Adipo−/− mice and WT littermates by repeatedly re-exposing the mice to the conditioning context daily without foot shock for 4 consecutive days.**
- **It has been suggested that extinction learning may be triggered by memory retrieval.**
- **To examine whether initial retrieval of fear memory after conditioning is affected in Adipo−/− mice, freezing responses during the first 3 min of extinction training session E1 was analyzed.**
- **Mice of both genotypes displayed high levels of freezing responses on E1 and showed no genotype difference (Figure 1a).**
- **Adipo−/− mice exhibited a significantly slower time course of contextual fear extinction, as compared with WT littermates (Figure 1a).**

**Facilitation of contextual fear extinction by intra-DG infusions of adiponectin**

- **Given the abundant expression of AdipoR1 and AdipoR2 in the DG of the hippocampus and the critical role of this brain region in contextual fear extinction,** we reasoned that the DG could be a site mediating the effects of adiponectin on contextual fear extinction. To test this possibility, mice were bilaterally cannulated in the DG and subjected to contextual fear conditioning (Figure 1b). Fear-conditioned mice received an intra-DG infusion (0.25 μg) per day for 3 days before extinction training. Over the course of 4-day extinction training, adiponectin was infused into the DG 30 min before each extinction session (Figure 1b). Initial fear retrieval during the first 3 min of extinction session E1 showed no difference between treatment groups (Figure 1b).
- **Mice that received intra-DG infusions of adiponectin showed accelerated fear-extinction learning, spending less time in freezing during extinction sessions 2 and 3 than vehicle-treated mice (Figure 1b).**
- **These results suggest that adiponectin in the DG facilitates extinction of contextual fear.**
Modulation of intrinsic excitability of DG granule neurons by adiponectin

To explore the cellular mechanisms of adiponectin modulation of contextual fear extinction, we examined whether adiponectin regulates excitability of DG granule neurons. First, whole-cell current clamp recordings were performed on DG granule neurons to examine the effects of adiponectin deficiency on their intrinsic excitability properties in response to depolarizing current injection (300 ms). DG granule neurons from Adipo−/− mice were hyperexcitable (Figure 2a), exhibiting a greater number of action potentials in response to current injections ranging from 45 to 90 pA (Figure 2a1), lower rheobase current (Figure 2a2) and higher input resistance (Figure 2a4) than DG neurons from WT littermates. Other membrane properties including resting membrane potential, action potential threshold and fAHP amplitude, half-width and amplitude of action potentials did not significantly differ between genotypes (Figures 2a3 and 2a5, and Supplementary Table 1).

Next, we tested whether adiponectin acutely modulates intrinsic excitability of DG granule neurons. A baseline recording of DG granule cells from WT mouse brain slices was first made followed by bath application of 15 μM AdipoRon, an AdipoR agonist that binds to both AdipoR1 and AdipoR2 with comparable high affinities.10 In response to depolarizing current injections (300 ms), AdipoRon treatment decreased the excitability of DG granule neurons, causing reduced number of action potentials elicited by current injections (60–90 pA) (Figure 2b1), increased rheobase current (Figure 2b2) and a more negative resting membrane potential (Figure 2b3) without changing other membrane properties including input resistance, action potential threshold, fAHP and action potential waveforms (Figures 2b4 and Supplementary Table 2). To determine whether hyperexcitability of DG neurons in Adipo−/− mice can be reversed by AdipoRon, we examined the effects of bath application of AdipoRon. AdipoRon significantly decreased the number of action potentials (Figure 2c1), increased rheobase (Figure 2c2) and caused the resting membrane potential to be more negative (Figure 2c3) without changing other membrane properties (Figures 2c4 and Supplementary Table 2), similar to those observed in WT mice. These data suggest that AdipoRon acutely suppress the excitability of DG granule neurons.
Normal contextual fear conditioning and extinction in mice lacking AdipoR1 in the DG.

To determine what role AdipoR1 has in the formation and extinction of contextual fear, we generated AdipoR1 floxed mice with conditional alleles carrying loxp sites inserted using a gene-targeting strategy (Figure 3a). The AdipoR1<sup>fox/fox</sup> mice were cross-bred with POMC-Cre mice to generate AdipoR1<sup>POMC-Cre</sup> mice. POMC-Cre-mediated excision was detected primarily in the DG in Ai14 tdTomato reporter mice (Figure 3b). Deletion of AdipoR1 exon 2 was confirmed by reverse transcriptase-PCR in dissected DG tissue from AdipoR1<sup>POMC-Cre</sup> mice (Figure 3b). The deletion of exon 2 led to a frame shift and the generation of a null mutant of AdipoR1. AdipoR1<sup>POMC-Cre</sup> mice were similar to WT littermate controls in freezing responses during contextual fear conditioning. There was no difference in freezing responses during the first 3 min of the initial extinction training E1 and the freezing levels over the 4-day extinction training (E1–4) were comparable between AdipoR1<sup>POMC-Cre</sup> mice and WT littermate controls (Figure 3b). Mice were then given two additional days of extinction training, in order to determine whether a greater number of training sessions might reveal some effects of AdipoR1 conditional knockout. However, no significant difference in freezing responses was observed between the two genotypes following extended training sessions (ES–6) (Figure 3b).

The possibility of compensatory changes during development could mask a phenotype and produce false-negative results. To rule out this possibility, we used AAV-Cre-GFP to induce deletion of AdipoR1 in the DG in adult mice. AdipoR1<sup>fox/fox</sup> mice received bilateral intra-DG injection of AAV-Cre-GFP or AAV-GFP. Three weeks after AAV injection in the DG, mice were subjected to contextual fear conditioning followed by extinction training for 6 days. There were no detectable differences in the acquisition, retrieval and extinction between AAV-Cre-GFP- and AAV-GFP-injected mice (Figure 3c). AAV-Cre-mediated knockdown of AdipoR1 in the DG was confirmed using western blotting 3 weeks after virus injection in AdipoR1<sup>fox/fox</sup> mice (Supplementary Figure 2).

**AdipoR2 knockout mice exhibit abnormal contextual fear responses**

To study the role of AdipoR2 in contextual fear memory, AdipoR2 knockout (AdipoR2<sup>−/−</sup>) mice and WT littermates received contextual fear-conditioning training followed by 4 days of extinction training (one session per day). AdipoR2<sup>−/−</sup> mice exhibited normal freezing responses during contextual fear conditioning but exaggerated freezing responses to the conditioning context during the first 3 min of extinction training session E1 (Figure 4a). Increased freezing levels were sustained across the extinction training sessions (Figure 4a). Owing to higher freezing responses in the first session of extinction (E1) in AdipoR2<sup>−/−</sup> mice compared with WT littermate controls, freezing levels were normalized to that of extinction session E1 for each group.

**AdipoR2 knockout mice show lower contextual fear extinction**

AdipoR2<sup>−/−</sup> mice showed a slower rate of contextual fear extinction over the 4-day course of extinction training (Figure 4a). These results indicate that AdipoR2 knockout leads to enhanced and sustained expression of contextual fear but impaired extinction learning. These behavioral changes were not associated with increased anxiety measured in the elevated plus maze test (Supplementary Figure 3a) or caused by abnormal nociceptive responses and visual function (Supplementary Figure 3b,c).

**Intra-DG adiponectin fails to facilitate contextual fear extinction in AdipoR2 knockout mice**

To address whether AdipoR2 mediates the effects of intra-DG adiponectin on contextual fear extinction, AdipoR2<sup>−/−</sup> mice were bilaterally cannulated in the DG and subjected to the same contextual fear-conditioning and extinction-training paradigms as did in the study of intra-DG adiponectin injection in WT mice. In addition, the WT littermates of AdipoR2<sup>−/−</sup> mice were included as controls for stereotaxic cannulation and microinjection procedures. Chronically cannulated AdipoR2<sup>−/−</sup> mice and their WT littermate controls exhibited similar freezing levels during contextual fear conditioning (Figure 4b). Subsequently, fear-conditioned mice received intra-DG infusions of adiponectin or vehicle for 3 days. AdipoR2<sup>−/−</sup> mice that received either intra-DG adiponectin or intra-DG vehicle showed higher freezing levels in response to the conditioning context during the first 3 min of extinction training session E1, as compared with vehicle-infused WT littermate controls (Figure 4b). AdipoR2<sup>−/−</sup> mice exhibited higher freezing levels than WT controls during extinction training and showed no difference between adiponectin- and vehicle-treated groups (Figure 4b). Furthermore, freezing levels were normalized to that of the first extinction session E1 for each group. The rate of extinction of adiponectin-infused AdipoR2<sup>−/−</sup> mice was comparable to that of vehicle-infused AdipoR2<sup>−/−</sup> mice (Figure 4b). These results indicate that AdipoR2<sup>−/−</sup> mice are resistant to the effects of intra-DG adiponectin on contextual fear extinction.

**AdipoR2 knockout mice display hyperexcitability of DG granule neurons**

Next, we determined whether AdipoR2 knockout affects intrinsic excitability of DG granule neurons. As observed in AdipoR1<sup>−/−</sup> mice, DG granule neurons from AdipoR2<sup>−/−</sup> mice were hyperexcitable in response to depolarizing current injections (300 ms) (Figure 5a), displaying a greater number of action potentials (Figure 5a1), lower rheobase (Figure 5a2) and higher input resistance (Figure 5a4) than DG neurons from WT littermates. In addition, AdipoR2<sup>−/−</sup> DG neurons showed a lower action potential threshold (Figure 5a5). Resting membrane potential (Figure 5a3), fAHP and the half-width and amplitude of action potentials did not differ significantly between the genotypes (Supplementary Table 1).

**Figure 2.** Adiponectin modulates intrinsic excitability of dentate gyrus (DG) granule neurons. (a1) Left, action potential (AP) trains induced by injecting a 90-pA current. The shaded area indicating the first 300 ms. Right, number of APs (genotype: F<sub>1(19)</sub> = 5.484, P = 0.03; current: F<sub>6(114)</sub> = 27.15, P < 0.001; genotype x current interaction: F<sub>6(114)</sub> = 3.113, P = 0.007); (a2) rheobase current (t<sub>19</sub> = 1.873, P = 0.076); (a3) resting membrane potential (t<sub>19</sub> = 0.175, P = 0.863); (a4) input resistance (t<sub>19</sub> = 2.632, P = 0.015); (a5) AP threshold (t<sub>10</sub> = 0.649, P = 0.496 from 3 wild-type (WT) mice; n = 11 from 3 AdipoR1<sup>−/−</sup> mice. *P < 0.05, **P = 0.076, ***P < 0.01 and ****P < 0.001 compared with WT littermates. (b) Decreased excitability of DG neurons in WT mice after bath application of 15 μM Adiporon. (b1) Left, action potential trains elicited by injecting a 90-pA current. Right, number of AP (treatment: F<sub>1(8)</sub> = 8.757, P = 0.016; current: F<sub>6(50)</sub> = 34.61, P < 0.001; treatment x current interaction: F<sub>6(300)</sub> = 7.338, P < 0.001). (b2) rheobase current (t<sub>10</sub> = 2.449, P = 0.036); (b3) resting membrane potential (t<sub>10</sub> = 3.496, P = 0.0068); (b4) input resistance (t<sub>10</sub> = 1.533, P = 0.159); (b5) AP threshold (t<sub>10</sub> = 0.235, P = 0.819). n = 10 from 4 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with artificial cerebrospinal fluid (aCSF) treatment. (c) Decreased excitability of DG neurons in AdipoR1<sup>−/−</sup> mice by bath application of 15 μM Adiporon. (c1) Left, action potential trains induced by injecting a 90-pA current. Right, number of APs (treatment: F<sub>1(8)</sub> = 8.475, P = 0.02; current: F<sub>6(48)</sub> = 24.30, P < 0.001; treatment x current interaction: F<sub>6(48)</sub> = 5.846, P < 0.001); (c2) rheobase current (t<sub>10</sub> = 3.500, P = 0.0081); (c3) resting membrane potential (t<sub>10</sub> = 5.487, P = 0.0006); (c4) input resistance (t<sub>10</sub> = 1.688, P = 0.130); (c5) AP threshold (t<sub>10</sub> = 1.294, P = 0.232). n = 9 from 4 mice. **P < 0.01 and ***P < 0.001 compared with aCSF treatment. Error bars represent s.e.m.
AdipoRon fails to modulate intrinsic excitability of DG granule neurons in AdipoR2 knockout mice

To determine whether AdipoR2 mediates the inhibitory effect of adiponectin on intrinsic excitability of DG granule neurons, DG granule cells from AdipoR2−/− mouse brain slices was first recorded under baseline, followed by bath application of 15 μM AdipoRon. AdipoRon failed to induce any significant change in intrinsic excitability of DG granule neurons (Figure 5b1). Intrinsic membrane properties including rheobase, resting membrane potential, input resistance and action potential threshold (Figures 5b2), as well as fAHP and action potential waveforms were not significantly altered by AdipoRon (Supplementary Table 2).
Together, these data indicate that AdipoR2 is required for adiponectin actions on DG granule neuron excitability.

**DISCUSSION**

Adipose tissue is now considered as an active and important endocrine organ, secreting a number of protein hormones (adipokines) into the circulation. Adiponectin is the most abundant adipokine in the circulation and can cross the blood-brain barrier to target neurons directly, thus presumably participating in various brain functions under physiological and pathological states. In this study, we report the identification of a novel role for adiponectin in fear memories. Adiponectin deficiency did not affect acquisition of contextual fear but impaired extinction learning. By contrast, intra-DG infusions of adiponectin facilitated contextual fear extinction. Deletion of AdipoR2, but not AdipoR1, enhanced fear expression and resulted in resistance to intra-DG adiponectin-induced facilitation of fear extinction. Furthermore, the cellular mechanisms underlying behavioral changes may involve modulation of intrinsic excitability of DG granule neurons. We believe these findings provide the first evidence that adiponectin and AdipoR2 have important roles in the regulation of fear memories and DG neuronal excitability.

Our previous studies have shown that adiponectin insufficiency increases susceptibility to stress-induced depressive behaviors,
Adiponectin, fear extinction and DG excitability

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Figure 5. AdipoR2 deletion enhances intrinsic excitability of dentate gyrus (DG) granule neurons and abolishes the inhibitory effect of AdipRon. (a1) Enhanced intrinsic excitability of DG granule neurons in AdipoR2−/− mice. (a1) Left, representative action potential trains induced by injecting a 90-pA current. The shaded area indicating the first 300 ms. Right, a greater number of action potentials was elicited by depolarizing current injection in AdipoR2−/− mice (genotype: F(1,27) = 7.257, P = 0.012; current: F(6,162) = 45.11, P < 0.001; genotype × current interaction: F(6,162) = 5.23, P < 0.001); (a2) rheobase current (t(27) = 2.631, P = 0.0139); (a3) resting membrane potential (t(27) = 1.790, P = 0.0846); (a4) input resistance (t(27) = 2.307, P = 0.029); (a5) action potential threshold (t(27) = 2.653, P = 0.0145). n = 14 from 4 wild-type (WT) mice; n = 15 from 4 AdipoR2−/− mice. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with WT. (b) Bath application of 15 μM AdipoRon failed to decrease excitability of DG granule neurons (n = 9 from 3 mice) in AdipoR2−/− mice. (b1) Left, action potential trains induced by injecting a 90-pA current. Right, number of action potentials (treatment: F(1,27) = 0.217, P = 0.654; current: F(6,162) = 40.26, P < 0.001; treatment × current interaction: F(6,162) = 0.545, P = 0.771); (b2) rheobase (t(20) = 0.359, P = 0.729); (b3) resting membrane potential (t(20) = 1.852, P = 0.101); (b4) input resistance (t(20) = 2.037, P = 0.076); (b5) action potential threshold (t(20) = 0.8162, P = 0.438). Error bars represent s.e.m.

including social defeat-induced social avoidance.33 The avoidance is also a common feature of PTSD. In this study, we used a classical Pavlovian fear-conditioning model, in which animals learn to associate a particular context with footshock. Extinction occurs when the animal is repeatedly exposed to the context without footshock after conditioning.38–50 Extinction is thought to be a new learning process to form ‘inhibitory association’ between the context and footshock.49–51 Using this paradigm, we found that mice deficient for adiponectin exhibited normal contextual fear conditioning but impaired extinction learning. These data suggest that adiponectin is necessary for extinction but not for acquisition of contextual fear memory. By contrast, infusions of adiponectin directly into the DG before extinction training facilitated extinction of contextual fear, suggesting that increasing adiponectin levels in the DG is sufficient to accelerate fear extinction. Because of high levels of expression of both AdipoR1 and AdipoR2 in the DG, the question was then raised as to which adiponectin receptor subtype(s) mediate the effects of adiponectin on contextual fear memories. AdipoR1 is widely distributed in the brain,33 therefore, we generated AdipoR1 flox mice in order to achieve neuron-
and/or region-specific gene deletion. We found that deletion of AdipoR1 from DG neurons had little or no effect on acquisition, retrieval and extinction of contextual fear. This is unlikely due to developmental compensation/adaptation, as similar results were obtained from mice with AAV-Cre-mediated deletion of AdipoR1 in adult DG. In contrast to AdipoR1, expression of AdipoR2 is restricted largely to the DG with weak-to-moderate levels in a few other brain regions such as the hypothalamus. As AdipoR1 conditional knockout mice exhibited normal acquisition but increased retrieval/expression of contextual fear memories and slowed fear extinction rate. These behavioral phenotypes overlapped with, but were not identical to, those observed in mice deficient in adiponectin. Disruption of both AdipoR1 and AdipoR2 abolishes adiponectin binding, suggesting that they are the major receptors for adiponectin in vivo.

In vitro studies have reported that AdipoR1 and AdipoR2 are capable of forming homomers and heteromers, and have distinct adiponectin binding affinities and functional signaling preferences. Although AdipoR1 activates AMP-activated protein kinase, AdipoR2 induces peroxisome proliferator-activated receptor-α signaling. Interestingly, the presence of AdipoR1/AdipoR2 heteromers provokes a delay in the onset of adiponectin-induced AMP-activated protein kinase activation. Presumably, deletion of AdipoR2 would increase AdipoR1 homodimerization and improve AdipoR1 signaling on adiponectin stimulation. However, the lack of behavioral responses of AdipoR2 knockout mice to intra-DG adiponectin argues against a possible role of AdipoR1 in this region in mediating the effects of adiponectin, but supports the essential role of AdipoR2. One alternative explanation for the enhanced retrieval/expression of contextual fear in AdipoR2 knockout mice but not in adiponectin-deficient mice could be due to adiponectin action via AdipoR1 outside the DG counteracting its effect via AdipoR2 within the DG.

Fear conditioning and extinction have been shown to differentially modulate intrinsic neuronal excitability in several brain structures including the hippocampus. Pharmacological and genetic modifications of intrinsic excitability are associated with the strength of new learning. The intrinsic excitability of a neuron determines the output of that neuron, thus endowing the cell with a unique functional identity. The DG functions as a gate to filter out aberrant or excessive excitatory input to the hippocampus and the unique intrinsic membrane properties of granule neurons participate in governing resistance to excessive excitation. In order to understand the cellular mechanisms underlying adiponectin action on extinction learning and fear memories, we investigated the intrinsic membrane excitability of DG granule neurons in brain slices. We found that mice deficient in adiponectin displayed enhanced intrinsic excitability of DG granule neurons. Similar changes were also seen in DG granule neurons from AdipoR2 knockout mice. This intrinsic hyperexcitability in both mutant mouse lines was reflected by an increased number of action potentials evoked by the depolarizing intracellular current injection and was attributed to an increase in input resistance. A lower action potential threshold could also contribute to AdipoR2 knockout-induced intrinsic hyperexcitability. In contrast, acute bath application of AdipoRon, a small-molecule adiponectin mimic, decreased intrinsic excitability of DG granule neurons. However, such effect was not caused by a decrease in input resistance but by a more negative resting membrane potential. Together, these results suggest that the opposing effects of loss of endogenous adiponectin/AdipoR2 and acute AdipoRon agonist treatment on DG neuronal excitability are mediated via modulating distinct intrinsic membrane properties. Although the underlying molecular mechanisms remain to be investigated, the increase in input resistance observed in adiponectin and AdipoR2 knockout mice could be the result of a decrease in total membrane surface area. This notion is supported by our recent study, demonstrating a reduction in dendritic branching and length in DG granule neurons of adiponectin-deficient mice (D. Zhang, X. Wang and X.Y. Lu, unpublished observations) and the evidence for a functional link between dendritic structural degeneration and neuronal hyperexcitability. However, a change in total membrane surface area of DG neurons is unlikely to occur following acute AdipoRon application in minutes. This might explain the unaltered input resistance in response to AdipoRon. Instead, AdipoRon was found to induce hyperpolarization of the resting membrane potential, consequently rendering the neuron less excitable. The possible mechanisms may involve modulating voltage-independent leak potassium channels, which are essential for establishing the resting membrane potential. Supporting evidence comes from the report showing that adiponectin hyperpolarizes a subset of hypothalamic neurons through the activation of voltage-independent potassium current. Although AdipoR2 knockout and AdipoRon treatment affected excitability of DG granule neurons via different modifications of intrinsic membrane properties, AdipoR2 knockout abolished the effect of AdipoRon. This is consistent with the absence of behavioral responses of AdipoR2 knockout mice to intra-DG infusions of adiponectin. The modulation of intrinsic excitability may explain the behavioral alterations observed in mice deficient in adiponectin or AdipoR2 and following intra-DG administration of adiponectin after fear conditioning. Although it is currently unknown whether fear conditioning and extinction modulate intrinsic excitability of DG granule neurons, studies have shown that DG neurons are activated during contextual fear conditioning and on re-exposure to the conditioning context. Optogenetic reactivation of DG neurons that are activated during contextual fear conditioning is sufficient to drive the recall of fear memory. Based on our data, we hypothesize that enhancing or dampening DG neuronal activation evoked by a fear-inducing context would cause resistance to or acceleration of fear extinction. However, this hypothesis requires further investigation in future studies.

In summary, our results support a functional link between adiponectin/AdipoR2 activation, intrinsic excitability of DG granule neurons and extinction of contextual fear. Exposure-based therapy for PTSD is thought to rely on extinction mechanisms. However, this therapy produces only temporary PTSD remission. Enhancing the formation and retention of extinction memory will therefore be critical to solving this challenge. Our current findings suggest that increasing adiponectin production or activating AdipoR2 receptors might be useful for facilitating extinction-based exposure treatments for PTSD and other trauma- and stress-related disorders.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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