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Zonisamide upregulates neuregulin-1 expression and enhances acetylcholine receptor clustering at the *in vitro* neuromuscular junction

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ABSTRACT

Decreased acetylcholine receptor (AChR) clustering compromises signal transmission at the neuromuscular junction (NMJ) in myasthenia gravis, congenital myasthenic syndromes, and motor neuron diseases. Although the enhancement of AChR clustering at the NMJ is a promising therapeutic strategy for these maladies, no drug is currently available for this enhancement. We previously reported that zonisamide (ZNS), an anti-epileptic and anti-Parkinson's disease drug, enhances neurite elongation of the primary spinal motor neurons (SMNs). As nerve sprouting occurs to compensate for the loss of AChR clusters in human diseases, we examined the effects of ZNS on AChR clustering at the NMJ. To this end, we established a simple and quick co-culture system to reproducibly make *in vitro* NMJs using C2C12 myotubes and NSC34 motor neurons. ZNS at 1–20 μM enhanced the formation of AChR clusters dose-dependently in co-cultured C2C12 myotubes but not in agrin-treated single cultured C2C12 myotubes. We observed that molecules that conferred responsiveness to ZNS were not secreted into the co-culture medium. We found that 10 μM ZNS upregulated the expression of neuregulin-1 (*Nrg1*) in co-cultured cells but not in single cultured C2C12 myotubes or single cultured NSC34 motor neurons. In accordance with this observation, inhibition of the Nrg1/ErbB signaling pathways nullified the effect of 10 μM ZNS on the enhancement of AChR clustering in *in vitro* NMJs. Although agrin was not induced by 10 μM ZNS in co-cultured cells, anti-agrin antibody attenuated ZNS-mediated enhancement of AChR clustering. We conclude that ZNS enhances agrin-dependent AChR-clustering by upregulating the Nrg1/ErbB signaling pathways in the presence of NMJs.

1. Introduction

Defective signal transmission at the neuromuscular junction (NMJ) due to decreased clustering of acetylcholine receptors (AChR) is observed in myasthenia gravis (MG) (Ohno et al., 2017), congenital myasthenic syndromes (CMS) (Ohno et al., 1996; Engel et al., 1999), and motor neuron diseases (Fischer et al., 2004; Monani, 2005; Boillee et al., 2006; Gould et al., 2006). Cholinesterase inhibitors to increase the acetylcholine (ACh) concentration in the synaptic cleft and 3,4-diaminopyrdine to increase ACh release from the nerve terminal ameliorate the defective signal transmission at the NMJ. No therapy, however, is currently aimed at increasing AChR clustering at the NMJ.

In an effort to identify a drug that enhances the NMJ signal transmission and to analyze the molecular mechanisms of the NMJ formation, *in vitro* NMJs have been generated using combinations of myotubes and spinal motor neurons (SMNs) derived from primary cells (Kobayashi and Askanas, 1985; Bonner et al., 1994; Mars et al., 2000; Das et al., 2007; Morimoto et al., 2013; Vilmont et al., 2016), embryonic stem cells (Soundararajan et al., 2007; Umbach et al., 2012), and induced pluripotent stem cells (iPSCs) (Stockmann et al., 2013; Osaki et al., 2018). Most of these systems required complicated procedures to make

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Abbreviations

ACh	Acetylcholine
AChR	acetylcholine receptor
α-BTX	α-bungarotoxin
CCM	co-culture medium
CMS	congenital myasthenic syndrome
DM	differentiation medium
GM	growth medium
GDNF	glial cell line-derived neurotrophic factor
iPSC	induced pluripotent stem cell
LRP4	low-density lipoprotein receptor-related protein 4
MG	myasthenia gravis
MuSK	muscle-associated receptor tyrosine kinase
Nrg1	neuregulin-1
NMJ	neuromuscular junction
Rspo2	R-spondin 2
PD	Parkinson's disease
SMNs	spinal motor neurons
ZNS	zonisamide.

myotubes, SMNs, and *in vitro* NMJs. Cell line-derived *in vitro* NMJs were generated by C2C12 myotubes and NG108-15 neuronal cells, which, however, required more than two weeks to make *in vitro* NMJs (Fu et al., 1997).

SMNs-derived secreted proteins induce AChR clustering and subsynaptic gene expressions especially *Chrne*, encoding the AChR ε subunit, during the NMJ synaptogenesis (Ohno et al., 2017; Ohkawara et al., 2021). Agrin (Burden, 2011) and neuregulin-1 (Nrg1) (Rimer et al., 1998) are well characterized secreted proteins at the NMJs. Agrin and Nrg1 are large molecules that are also classified as extracellular matrix proteins, and are likely to be anchored at the NMJ immediately after secretion. Neuronal agrin isoform induces AChR clustering (Jones et al., 1996; Meier et al., 1997) and *Chrne* expression via its receptors, LRP4 and MuSK, in the skeletal muscle (Valenzuela et al., 1995; Kim et al., 2008; Zhang et al., 2008). Nrg1, which has multiple spliced isoforms in SMNs and skeletal muscle, also induces the formation of AChR clusters at the NMJ via ErbB receptors (Rimer, 2007; Schmidt et al., 2011; Ngo et al., 2012). We and others also reported four additional small secreted proteins that enhanced the NMJ synaptogenesis: GDNF (Zahavi et al., 2015), R-spondin 2 (Rspo2) (Nakashima et al., 2016), fibroblast growth factor 18 (Fgf18) (Ito et al., 2018), and connective tissue growth factor (Ctgf) (Ohkawara et al., 2020).

Zonisamide (ZNS) is clinically used in both monotherapy and adjunctive therapy for children and adults with generalized or partial seizures (Brigo et al., 2018). ZNS is also clinically used as an anti-Parkinsonian disease (PD) agent particularly for resting tremor (Murata, 2004). We previously reported that ZNS enhanced neurite elongation of primary SMNs, and facilitated nerve regeneration of an autograft of the sciatic nerve in mice (Yagi et al., 2015; Ohno et al., 2016). We also showed that ZNS was protective against the loss of SMNs in a rat model of compressive cervical myelopathy (Kanbara et al., 2020) and suppresses neuroinflammation in a mouse model of neuropathic pain (Koshimizu et al., 2020).

In the current study, we made a protocol to reproducibly make *in vitro* NMJs using C2C12 myotubes and NSC34 motor neurons. As nerve sprouting to increase the number of NMJs is observed in MG and CMS (Ohno et al., 2017), and as NMJ-specific genes were upregulated in a mouse model of nerve autograft (Yagi et al., 2015), we examined the effects of ZNS on *in vitro* NMJs. We report that ZNS enhances the expression of Nrg1 in co-cultured cells but not in single cultured C2C12 myotubes or single cultured NSC34 motor neurons, and facilitates AChR clustering in an agrin-dependent manner.

2. Materials and methods

2.1. Single culture of C2C12 myotubes and NSC34 neuronal cells

C2C12 cells, an immortalized mouse myoblast cell line, was purchased from ATCC (CRL-1772). C2C12 cells were seeded at 100 cells/mm^2 on a 12-well or 6-well plate coated with 50 μg/mL collagen 1 (CORNING, 354236). One day after the passage (Day 1), the medium was changed to differentiation medium (DM), which was constituted of DMEM supplemented with 2% horse serum (Gibco, 1998112) and 1% Pen-Strep. Two days after adding DM (Day 3), the medium was changed to co-culture medium (CCM), which was constituted of DMEM/F12 (Gibco) supplemented with 0.5% horse serum, 1% non-essential amino acid (MP Biomedicals, 1681049), and 1% Pen-Strep with or without neuronal agrin (10 ng/mL, R&D Systems, AZH1216091) (Nakashima et al., 2016). CCM was changed every 2 days. Six days after starting CCM (Day 9), AChR clusters and gene expressions in single cultured C2C12 myotubes were evaluated.

NSC34 cells, a mouse neuroblastoma-SMN hybrid cell line, with a multipolar motor neuron-like phenotype stably express the doxycycline-induced green fluorescent protein (GFP) (NSC34-pTetR12-TO/GFP) (Cashman et al., 1992). The NSC34 cells were kindly provided by Dr. Shinsuke Ishigaki, Department of Neurology, Nagoya University Graduate School of Medicine. The cells were grown in growth medium (GM), which was constituted of DMEM supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, 172012) and 1% Pen-Strep (10,000 U/mL, Gibco, 151400-122). NSC34 cells were seeded at 100 cells/mm^2 on a 6-well plate coated with 0.001% poly L-lysine solution (PLL, Sigma-Aldrich, RNBG7150) with CCM. Six days after the plating, the gene expressions of single cultured NSC34 motor neurons were evaluated.

2.2. Co-culture of C2C12 myotubes and NSC34 neuronal cells

A co-culture plate was coated with 50 μg/mL collagen 1, as we performed for single cultured C2C12 myotubes. C2C12 cells (100 cells/mm^2) were cultured in GM for 1 day (from days 0–1) and then cultured in DM for 2 additional days (from days 1–3). On day 3, NSC34 cells were seeded at 50 cells/mm^2 on the partially differentiated C2C12 cells in CCM with or without 10 μM ZNS. CCM was changed every 2 days until being harvested on day 9. To inhibit the Nrg1/ErbB signaling, an ErbB2 inhibitor, AG879 (500 nM, Abcam, ab141059), and an ErbB4 inhibitor, AG1478 (500 nM, Abcam, ab141438) (Fukazawa, 2003) were added to CCM 6 h prior to harvesting cells. The suppressive effects of 5 μM AG879 and 5 μM AG1478 for 1–8 h on NMJ-related molecules in C2C12 and primary myotubes were previously reported (Schmidt et al., 2011). We reduced the concentrations of AG879 and AG1478 to 500 nM to retain the viability of co-cultured cells. To block agrin, anti-agrin antibody (80 ng/mL, R&D Systems, AF550) was added to CCM 2 days prior to harvesting cells according to the manufacturer's protocol. To examine the effect of exogenous Nrg-1, Nrg-1 (recombinant human neuregulin β-1, 3 nM, PEPROTECH, 1017G316J0317) (Ngo et al., 2012) was added to CCM 4 h prior to harvesting cells. On day 9, AChR clusters of C2C12 myotubes and mRNA expressions in co-cultured C2C12 myotubes and NSC34 motor neurons were quantitated.

2.3. Staining of AChR clusters in C2C12 myotubes

C2C12 myotubes were incubated with 10 μg/mL Alexa594-conjugated α-bungarotoxin (α-BTX, Invitrogen, 1938422). The cells were then fixed in 4% paraformaldehyde (PFA) for 15 min. Before and after fixation, the cells were washed with phosphate buffered saline (PBS) three times. For peripherin staining in addition to the α-BTX staining, the cells were treated with PBS containing 2% goat serum (Fujifilm Wako Pure Chemical, WDR2410) and 0.1% Triton X-100 for 1 h. After washing with PBS, the cells were incubated with rabbit

polyclonal anti-peripherin antibody (1:300, Millipore, 2972430) overnight at 4 °C, and with Alexa488-conjugated goat anti-rabbit IgG (1:100, Invitrogen, SH251139) for 1 h at room temperature. The staining was observed using an IX71 fluorescence microscope (Olympus) and analyzed by CellSens software (Olympus). AChR clusters were blindly evaluated by three metrics (the total area of AChR clusters, the total intensity of AChR clusters, and the length of AChR clusters) using MetaMorph software (Molecular Devices).

2.4. Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was isolated with the QuickGene RNA cultured cell kit (Kurabo) using QuickGene-800 (Kurabo). First-strand cDNA was synthesized with ReverTra Ace (Toyobo). mRNA expression levels were quantified using LightCycler 480 Real-Time PCR (Roche) and SYBR Green (Takara), and were normalized to that of β-2 microglobulin (*B2m*). The primer sequences are shown in Supplementary Table 1.

2.5. Evaluation of myotube twitching

C2C12 myotubes were cultured alone (see Supplementary Fig. S1A) or along with NSC34 cells (see Fig. 2A) on a 12-well plate with 0, 1, 10, 20 μM ZNS. On day 9, muscle twitching was observed under an IX71 fluorescence microscope (Olympus), and the total number of twitching myotubes in each well was blindly counted.

2.6. Statistical analysis

One-way or two-way analysis of variance (ANOVA) followed by post-hoc Tukey honest significant difference (HSD) test were performed using BellCurve for Excel (Social Survey Research Information Co., Ltd). *P*-values of 0.05 or less were considered statistically significant. Exact F-values and *p*-values of one-way or two-way ANOVA are indicated in the main text. A *p*-value less than 0.001 was indicated by $p < 0.001$ not by its exact value.

3. Results

3.1. Co-culture of C2C12 myotubes and NSC34 motor neurons induces AChR clusters in 9 days

In an effort to make a quick and reproducible *in vitro* NMJ model that can be applied to drug screening, we optimized co-culture protocols with mouse C2C12 skeletal myoblasts and mouse NSC34 neuroblastoma-SMN hybrid cells (Fig. 1A). To induce myotube differentiation, C2C12 cells were cultured for 1 day in GM and then cultured in DM for 2 days. Then the NSC34 cells were seeded on the partially differentiated C2C12 myotubes and subsequently cultured in CCM containing 0.5% horse serum and 1% non-essential amino acids for 6 days. We confirmed by phase contrast microscopy that C2C12 myotubes were sufficiently differentiated on day 9 (Fig. 1B). Staining for peripherin in the neurites and the neuronal bodies, as well as for AChR with α-BTX, showed that a small fraction of AChR clusters on C2C12 myotubes were juxtaposed to peripherin-positive neurites of NSC34 cells in this co-culture system (Fig. 1C).

3.2. Zonisamide (ZNS) increases the formation of AChR clusters, the expressions of NMJ-specific genes, and muscle twitching in a dose-dependent manner in in vitro NMJ

We previously showed that ZNS increased the neurite lengths and the number of branch points of NSC34 cells and mouse primary SMNs (Yagi et al., 2015; Ohno et al., 2016). As nerve sprouting compensates for defective AChR clusters in MG and CMS (Ohno et al., 2017), and as we previously reported that oral administration of ZNS increased the expressions of NMJ-specific *Chrne*, *Colq,* and *Rapsn* in the tibialis anterior in a mouse model of sciatic nerve autograft (Yagi et al., 2015), we investigated whether ZNS increases AChR clusters in *in vitro* NMJs (Fig. 2A). We found that the formation of AChR clusters was increased by ZNS on day 9 in a dose-dependent manner (area: $F(3,140) = 9.944$, $p < 0.001$; intensity: $F(3,140) = 8.954$, $p < 0.001$; length: $F(3,140) = 9.401$, $p < 0.001$; Fig. 2BC).

Next, we analyzed gene expressions of muscle differentiation markers and NMJ-specific molecules in co-cultured cells. MuSK (*Musk*), AChR ε subunit (*Chrne*), collagen Q (*Colq*), rapsyn (*Rapsn*), LRP4 (*Lrp4*), and β-catenin (*Ctnnb1*) play essential roles at the NMJ. MyoD (*Myod1*) and myosin heavy chain (*Myh1*) serve as muscle differentiation markers.

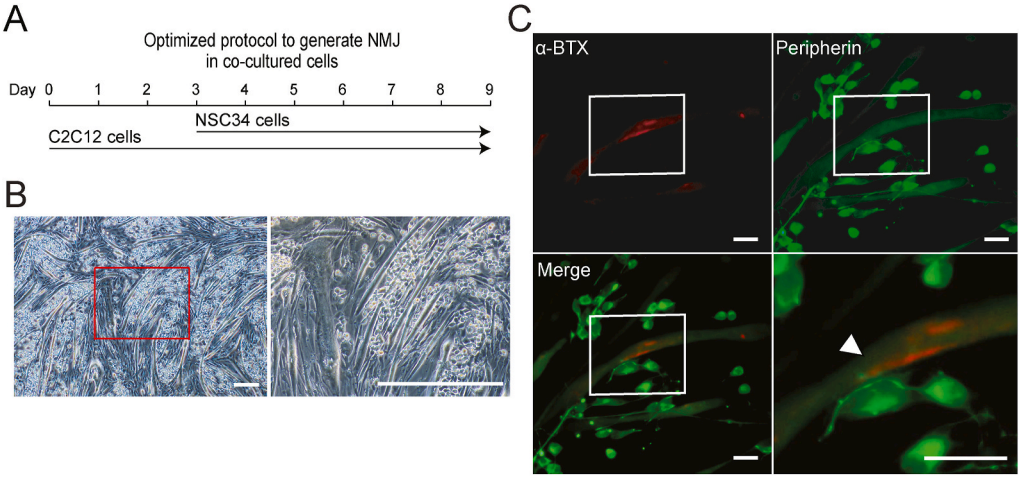

Fig. 1. Protocol and representative images of co-culture of C2C12 myoblasts/myotubes and NSC34 motor neurons. (A) Co-culturing protocol of C2C12 myoblasts/myotubes and NSC34 motor neurons. C2C12 myoblasts (100 cells/mm^2) were seeded in the growth medium for 1 day (days 0–1) and then cultured in the differentiation medium for 2 days (days 1–3). On day 3, the medium was changed to co-culture medium, and NSC34 cells (50 cells/mm^2) were seeded onto C2C12 myotubes. After co-culturing for 6 days (day 9), AChR clustering was evaluated. **(B)** Phase-contrast images in low (left) or high (right) magnification of co-cultured cells on day 9. Note that the differentiated C2C12 cells form myotubes. Round cells are mostly NSC34 motor neurons. Bar = 200 μm. **(C)** Co-cultured C2C12 myotubes and NSC34 motor neurons on day 9 were stained with Alexa594-conjugated α-BTX (red, upper left) and anti-peripherin antibody (green, upper right). NSC34 motor neurons have peripherin-positive neurites, and some of them end at C2C12 myotubes (merge, lower left) to form α-BTX-positive AChR clusters (arrowhead in the lower right magnified panel) on the myotubes. Bar = 50 μm.

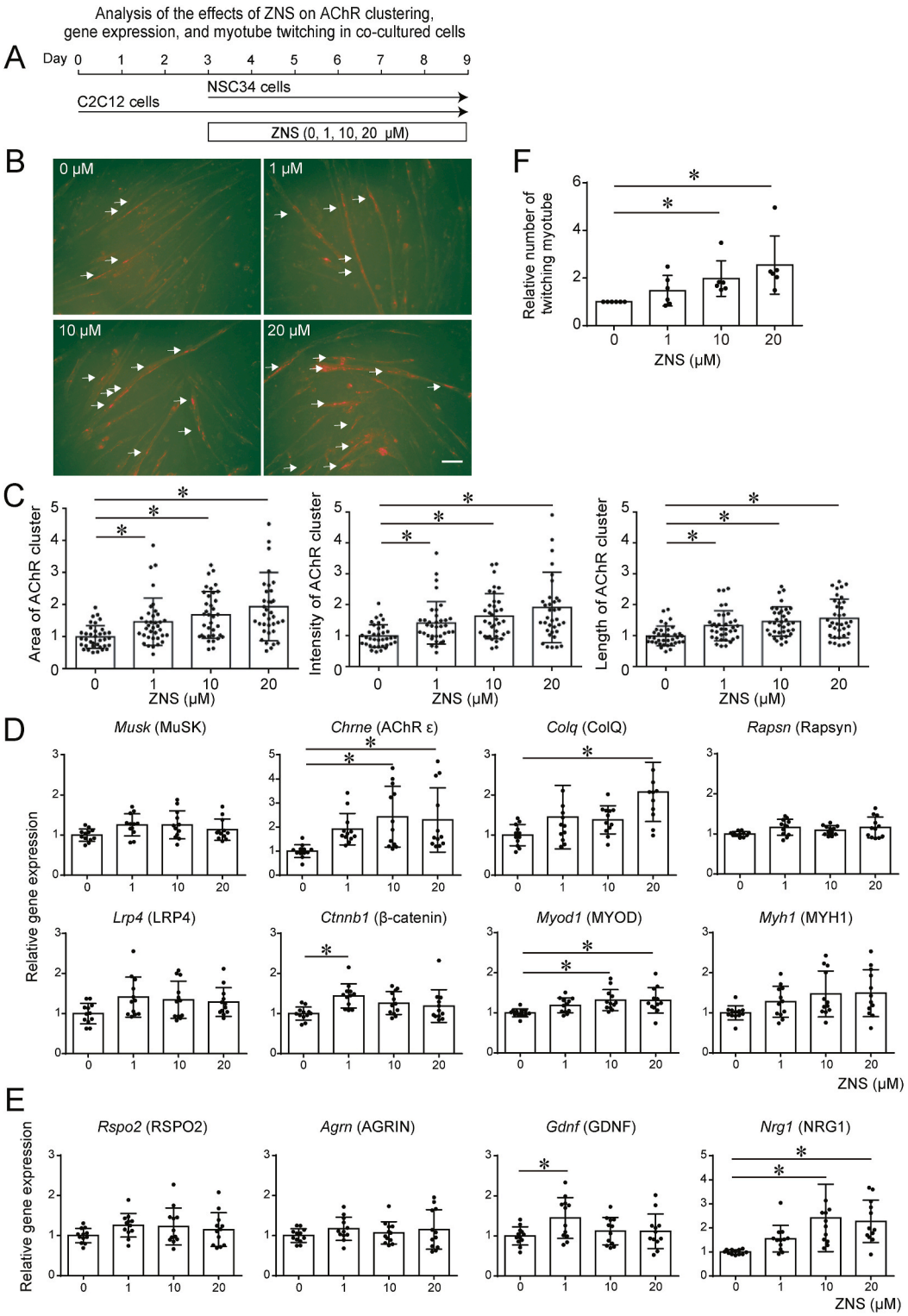

Fig. 2. Zonisamide (ZNS) enhances the formation of AChR clusters and the expressions of NMJ-related genes when C2C12 myotubes were co-cultured with NSC34 motor neurons. (A) Protocol to co-culture C2C12 myotubes and NSC34 motor neurons with 0, 1, 10, and 20 μM ZNS. On day 9, the cells were harvested to evaluate AChR clustering, mRNA expression, and muscle twitching. **(B, C)** AChR clusters on C2C12 myotubes with the indicated concentrations of ZNS were stained with α-BTX (red). Bar = 100 μm. Total area, total intensity, and total length of AChR clusters were blindly measured using MetaMorph software, and values were normalized to that without ZNS. Mean and SD are indicated ($n = 36$ optical fields in six wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD compared to 0 μM ZNS. **(D, E)** Quantitative RT-PCR to analyze expressions of NMJ-related genes and muscle differentiation markers **(D)**, and secreted proteins **(E)**, in co-cultured cells. Gene expression was normalized to that of *B2m* and then to the ratio without ZNS. Mean and SD are indicated ($n = 12$ wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD compared to 0 μM ZNS. **(F)** The number of twitching C2C12 myotubes was blindly counted. Values were normalized to that without ZNS. Mean and SD are indicated ($n = 6$ wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD compared to 0 μM ZNS.

In co-cultured cells, ZNS increased the expressions of *Chrne* ($F(3,44) = 5.156$, $p = 0.004$), *Colq* ($F(3,44) = 7.011$, $p < 0.001$), and *Myod1* ($F(3,44) = 4.974$, $p = 0.005$) in a dose-dependent manner, and *Ctnnb1* at 1 μM but not at 10 or 20 μM ($F(3,44) = 4.353$, $p = 0.009$; Fig. 2D). In contrast, no statistical significance was observed in *Musk*, *Rapsn*, or *Myh1*.

We also observed synchronized twitching of C2C12 myotubes co-cultured with NSC34 motor neurons on day 9. ZNS increased the number of twitching C2C12 myotubes in a dose dependent manner ($F(3,20) = 24.053$, $p < 0.001$; Fig. 2E). To summarize, in co-cultured cells, ZNS increased AChR clustering, induced the gene expressions of NMJ-specific molecules and muscle differentiation markers, and enhanced muscle twitching.

3.3. ZNS fails to increase the formation of AChR clusters, the expressions of NMJ-specific genes, and muscle twitching in single cultured C2C12 myotubes and single cultured NSC34 neuronal cells

To examine whether co-culture is required for the effects of ZNS, we analyzed the effects of ZNS in single cultured C2C12 myotubes (Supplementary Fig. S1A) and single cultured NSC34 motor neurons (Supplementary Fig. S1F). ZNS failed to increase α-BTX-positive AChR clusters (Supplementary Fig. S1BC), and did not increase gene expressions of *Musk, Chrne, Colq, Rapsn, Myh1, or Nrg1*, except for *Myod1* at 20 μM ZNS ($F(3,20) = 5.946$, $p = 0.005$; Supplementary Fig. S1D). Similarly, ZNS did not increase muscle twitching in single cultured C2C12 myotubes (Supplementary Fig. S1E). We also observed that ZNS treatment for 6 days did not increase either *Agrn* or *Nrg1* expression in single cultured NSC34 motor neurons (Supplementary Fig. S1F). These results suggest that ZNS required *in vitro* NMJ to exert its effects on AChR clustering and its associated features. We observed that AChR clustering (Fig. 2BC), *Colq* expression (Fig. 2D), and muscle twitching (Fig. 2F) were maximized at 20 μM, but the expressions of *Chrne* and *Nrg1* were saturated at 10 μM (Fig. 2DE). We and others previously reported the effect of ZNS on neurite elongation of NSC34 motor neurons (Yagi et al., 2015), primary spinal motor neurons (Yagi et al., 2015), and primary neurons from dorsal root ganglion (Takaku and Sango, 2020) at 10 μM. Although 20 μM ZNS might have more effects on the formation of

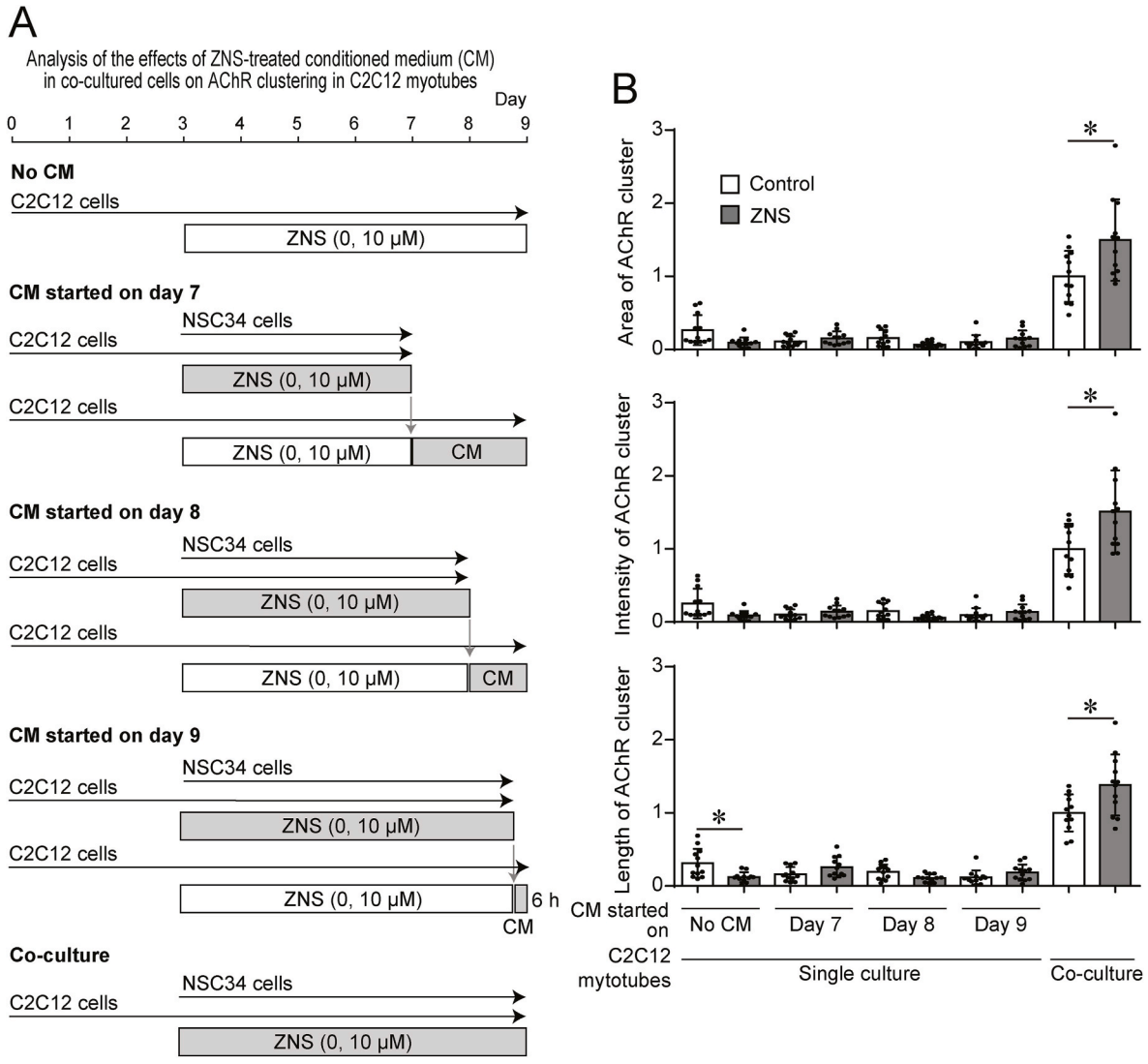

Fig. 3. Conditioned medium (CM) derived from co-cultured C2C12 myotubes and NSC34 motor neurons does not confer responsiveness to ZNS to single cultured C2C12 myotubes. (A) Protocols to transfer CM generated by co-cultured C2C12 myotubes and NSC34 motor neurons to single cultured C2C12 myotubes. CM was transferred on days 7 and 8, and at 6 h prior to harvesting cells on day 9. Cells were harvested on day 9. **(B)** AChR clusters in C2C12 myotubes were stained with α-BTX. Total area, total intensity, and total length of AChR clusters were blindly measured using MetaMorph software. Values were normalized to co-cultured cells without ZNS. Mean and SD are indicated ($n = 12$ optical fields in 2 wells each). *$p < 0.05$ by two-way ANOVA followed by Tukey HSD (only comparisons between ZNS 0 μM and 10 μM are indicated).

functional NMJ, we used 10 μM ZNS in the following experiments. We expected possible application of ZNS in clinical settings in the future, and hoped to reduce the concentration of ZNS as low as possible.

We next examined whether secreted molecules in the medium derived from co-cultured NSC34 motor neurons and C2C12 myotubes enabled ZNS to enhance AChR clustering. C2C12 myotubes were co-cultured with NSC34 cells and treated with or without ZNS from day 3, and the conditioned medium (CM) was harvested on days 7, 8, and 9 (Fig. 3A). On day 7, the CM was totally transferred to single cultured C2C12 myotubes that were cultured in parallel with the co-cultured C2C12 myotubes. AChR clustering was analyzed on day 9 after being exposed to the CM for 2 days. Similarly, on days 8 and 9, the CM was transferred to single cultured C2C12 myotubes, and AChR clustering was analyzed on day 9 after being exposed to the CM for 1 day and 6 h, respectively. We observed that AChR clustering was not increased in single cultured C2C12 myotubes that were exposed to the CM for 2 days, 1 day, or 6 h (Fig. 3B). These results suggest that the effects of ZNS on AChR clustering was not mediated by molecules secreted into the CM from co-cultured NSC34 motor neurons and C2C12 myotubes. The effects of ZNS, however, still can be mediated by secreted molecules that are embedded in the extracellular matrix or in the cell membrane. Alternatively, the effects of ZNS may be mediated by intracellular molecules that are induced by forming *in vitro* NMJs.

3.4. Agrin is required for ZNS-enhanced AChR clustering in in vitro NMJs, but does not mediate the effects of ZNS

Neuronal agrin isoform induces AChR clustering (Valenzuela et al., 1995; Kim et al., 2008; Zhang et al., 2008) and *Chrne* expression (Jones et al., 1996; Meier et al., 1997). We indeed observed that 10 μM of rat recombinant agrin induced AChR clustering (area: $F(3,92) = 62.639$, $p < 0.001$; intensity: $F(3,92) = 69.275$, $p < 0.001$; length: $F(3,92) = 90.878$, $p < 0.001$) and *Chrne* expression ($F(3,20) = 4.468$, $p = 0.016$) in single cultured C2C12 myotubes (Fig. 4ABC). Thus, ZNS may enhance *Agrn* expression to induce AChR clustering. We, however, found that ZNS did not increase *Agrn* expression in co-cultured cells (Fig. 2E) or single cultured NSC34 motor neurons (Supplementary Fig. S1F). We also examined the effects of ZNS on the other AChR cluster-inducing secreted proteins, Rspo2 (*Rspo2*) (Nakashima et al., 2016; Li et al., 2018) and GDNF (*Gdnf*) (Zahavi et al., 2015), which are expressed in both motor neurons and myotubes. ZNS did not increase the expression of *Rspo2*, but increased *Gdnf* at 1 μM but not at 10 or 20 μM ($F(3,44) = 2.908$, $p = 0.045$; Fig. 2E). We next examined whether ZNS requires agrin to exert its effect on AChR clustering using anti-agrin antibody. We first observed in co-cultured cells without ZNS that anti-agrin antibody suppressed AChR clustering (area: $F(3,92) = 29.509$, $p < 0.001$; intensity: $F(3,92) = 30.089$, $p < 0.001$; length: $F(3,92) = 19.248$, $p < 0.001$), but did not affect *Chrne* expression (Fig. 4DEF). Similarly, anti-agrin antibody attenuated the effects of ZNS on AChR clustering (area: $F(3,92) = 29.509$, $p < 0.001$; intensity: $F(3,92) = 30.089$, $p < 0.001$; length: $F(3,92) = 19.248$, $p < 0.001$) and *Chrne* expression ($F(3,20) = 19.453$, $p = 0.037$) in co-cultured cells (Fig. 4DEF). Although ZNS did not increase *Agrn* expression in co-cultured cells, ZNS might have enhanced the effect of agrin on C2C12 myotubes. To prove this hypothesis, we added agrin to single cultured C2C12 myotubes, and examined the effects of ZNS. ZNS, however, had no effect on AChR clustering or *Chrne* expression in agrin-treated C2C12 myotubes (Fig. 4ABC). These results suggest that agrin is required for ZNS-mediated enhancement of AChR clustering in *in vitro* NMJs, but is not sufficient to provide ZNS with an ability to enhance AChR clustering.

3.5. ZNS induces Nrg1 expression in in vitro NMJ, and requires the Nrg1/ErbB signaling to induce AChR clustering

Nrg1 is another secreted protein that is expressed in SMNs and skeletal muscle, and is involved in the formation of AChR clusters along with agrin at the NMJ (Rimer, 2007; Schmidt et al., 2011; Ngo et al., 2012). We first confirmed that Nrg1 alone could not enhance AChR clustering, but enhanced agrin-induced AChR clustering in single cultured C2C12 myotube (Supplementary Fig. S2AB). We then asked whether ZNS increases *Nrg1* expression. In contrast to the lack of upregulation of agrin expression by ZNS, ZNS induced *Nrg1* expression in co-cultured cells ($F(3,44) = 6.824$, $p < 0.001$; Fig. 2E), but not in single cultured C2C12 myotubes (Supplementary Fig. S1D) or single cultured NSC34 motor neurons (Supplementary Fig. S1F). *In vitro* NMJ is thus likely to be required for ZNS to induce Nrg1 expression. We next examined whether ZNS requires Nrg1 to exert its effect on AChR clustering. We first confirmed that Nrg1 enhanced the formation of AChR clusters and *Chrne* expression in co-cultured cells (Fig. 5ABC). We then examined the requirement of Nrg1 and its receptors, ErbBs, for the effects of ZNS. Nrg1 binds to ErbB3 and ErbB4 receptors (Zhu et al., 1995), but ErbB3 lacks tyrosine kinase activity and needs to be dimerized with ErbB2 or ErbB4 to deliver Nrg1 signaling (Carraway et al., 1994; Sliwkowski et al., 1994). We thus used a cocktail of an ErbB2 inhibitor (AG879) and an ErbB4 inhibitor (AG1478), which were previously reported to suppress the NMJ formation in C2C12 myotubes (Schmidt et al., 2011). ErbB inhibitors nullified the effects of ZNS on the enhancement of AChR clustering (area: $F(3,92) = 22.587$, $p < 0.001$; intensity: $F(3,92) = 21.001$, $p < 0.001$; length: $F(3,92) = 27.911$, $p < 0.001$) and the upregulation of *Chrne* expression ($F(3,20) = 7.740$, $p = 0.027$) in co-cultured cells (Fig. 5DEF). These results suggest that ZNS requires *in vitro* NMJ to induces *Nrg1* expression, and depends on the Nrg1/ErbB signaling to induce AChR clustering.

4. Discussion

We first established a simple and quick co-culturing protocol to reproducibly make *in vitro* NMJs in 9 days using C2C12 mouse myotubes and NSC34 SMN-neuroblastoma hybrid cells. Previously reported cell line-based *in vitro* NMJs used NG108-15 cells (a mouse neuroblastoma-rat glioma hybrid cell line) and C2C12 myotubes (Fu et al., 1997). NG108-15 cells were differentiated from 0 to 7 days. In parallel, C2C12 myoblasts were differentiated into myotubes for 2–10 days. The differentiated cells were mixed together and co-cultured for 3–10 days to make *in vitro* NMJs. These procedures took a total of 14 or more days. NSC34 motor neurons were previously paired with mouse primary muscle cells to make *in vitro* AChR clusters and to induce muscle twitching (Cashman et al., 1992). Primary muscle cells isolated from mouse embryos were differentiated for 5–6 days, and then co-cultured with NSC34 motor neurons for 4–5 days. The total procedures took more than 14 days. Although we aimed at drug screening, our *in vitro* AChR clusters with NSC34 motor neurons and C2C12 myotubes were generated faster than those of previous reports (Fig. 1).

In co-cultured cells, ZNS enhanced AChR clustering (Fig. 2BC), muscle twitching (Fig. 2F), and the expressions of *Nrg1* and NMJ-specific genes including *Chrne* (Fig. 2DE). In contrast, in single cultured C2C12 myotubes, ZNS had no effect on the formation of AChR clusters, muscle twitching, or the expressions of *Nrg1* and *Chrne* (Supplementary Fig. S1ABCDE). Similarly, in single cultured NSC34 motor neurons, ZNS had no effect on the expression of *Agrin* or *Nrg1* (Supplementary Fig. S1F). We also found that molecule(s) that conferred responsiveness to ZNS in co-cultured cells were not secreted into the culture medium (Fig. 3). Taken together, these results indicate that ZNS enhances AChR clustering only in the presence of communication between motor neurons and myotubes but not in each cell type alone (Fig. 5G). In addition, the effects of ZNS on the formation of AChR clusters and *Chrne* expression were dependent on agrin (Fig. 4ABC) and the Nrg1/ErbB signaling pathways (Fig. 5DEF). Nrg1/ErbB signaling stabilizes AChR clusters as well as postsynaptic apparatus including acetylcholinesterase, MuSK, and utrophin (Schmidt et al., 2011). Agrin induced AChR clustering without Nrg1, whereas Nrg1 required agrin to enhance AChR clustering (Supplementary Fig. S2), as has been reported previously (Ngo et al.,

2012). As ZNS increased the expression of *Nrg1* but not *Agrn* (Fig. 2E), the effects of ZNS on the enhancement of agrin-dependent AChR clustering may be achieved by Nrg1. The lack of the AChR cluster-inducing effect of ZNS in agrin-treated single cultured C2C12 myotubes (Fig. 4B) may be accounted for by the lack of induction of *Nrg1* expression by ZNS in single cultured C2C12 myotubes (Supplementary Fig. S1F). GDNF is a secreted protein that enhances axonal growth and innervation to form the NMJ (Zahavi et al., 2015). We previously reported that Rspo2 binds to Lgr5, and enhances LRP4/MuSK-mediated AChR clustering (Nakashima et al., 2016; Li et al., 2018). Although Rspo2 and GDNF were candidate molecules that might work with Nrg1 to confer the responsiveness to ZNS, *Rspo2* was not induced by ZNS and *Gdnf* was upregulated only at 1 μM ZNS but not at 10 or 20 μM ZNS in co-cultured cells (Fig. 2E). Thus, the dose-dependent induction of AChR clustering by ZNS

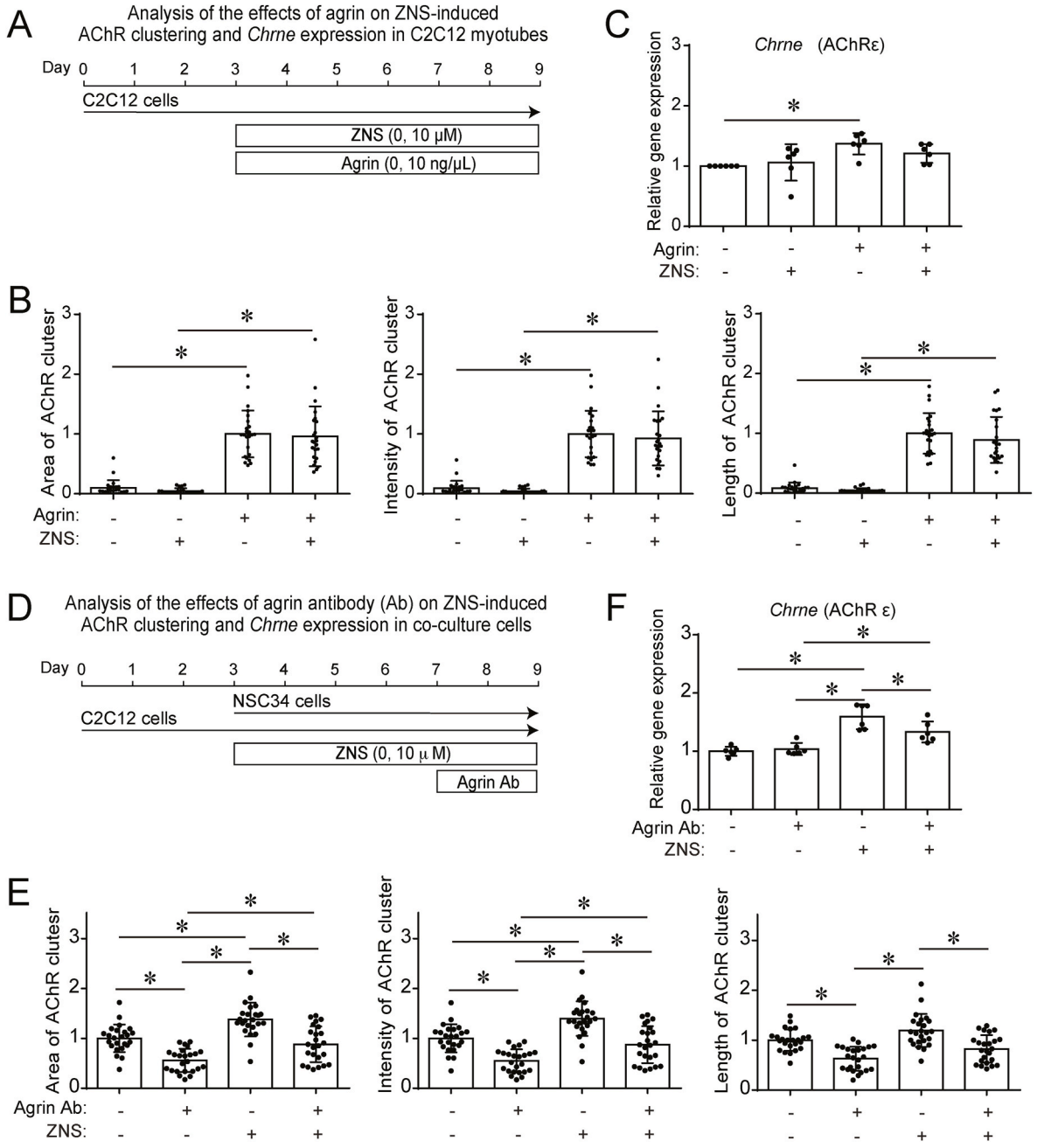

Fig. 4. Agrin signaling are not sufficient but required for ZNS to enhance AChR clustering and *Chrne* expression in C2C12 myotubes. **(A)** Protocol to culture C2C12 myoblasts/myotubes alone without NSC34 cells with ZNS (0 or 10 μM) and neural agrin (0 or 10 ng/mL). **(B)** AChR clusters on C2C12 myotubes were stained with α-BTX. Total area, total intensity, and total length of AChR clusters were blindly measured using MetaMorph software, and values were normalized to that with 10 ng/mL agrin alone. Mean and SD are indicated ($n = 24$ optical fields in six wells each). **(C)** *Chrne* expression was analyzed by quantitative RT-PCR. Gene expression was normalized to that of *B2m* and then to the ratio with 10 ng/mL agrin alone ($n = 8$ wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD. **(D)** Protocol to co-culture C2C12 myotubes and NSC34 motor neurons with 0 or 10 μM ZNS. Two days prior to harvesting cells, 80 ng/mL anti-agrin antibody (Agrin Ab) was added to the co-cultured medium. **(E)** AChR clusters in co-cultured C2C12 myotubes were stained with α-BTX (red). Total area, total intensity, and total length of the AChR clusters were blindly measured using MetaMorph software. Values were normalized to that of co-cultured ZNS-free C2C12 myotubes without anti-agrin antibody. Mean and SD are indicated ($n = 24$ optical fields in 4 wells each). **(F)** Quantitative RT-PCR to analyze *Chrne* expression in co-cultured C2C12 myotubes. Gene expression was normalized to that of *B2m,* and then to the ratio of ZNS-free cells without anti-agrin antibody. Mean and SD are indicated ($n = 6$ wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD.

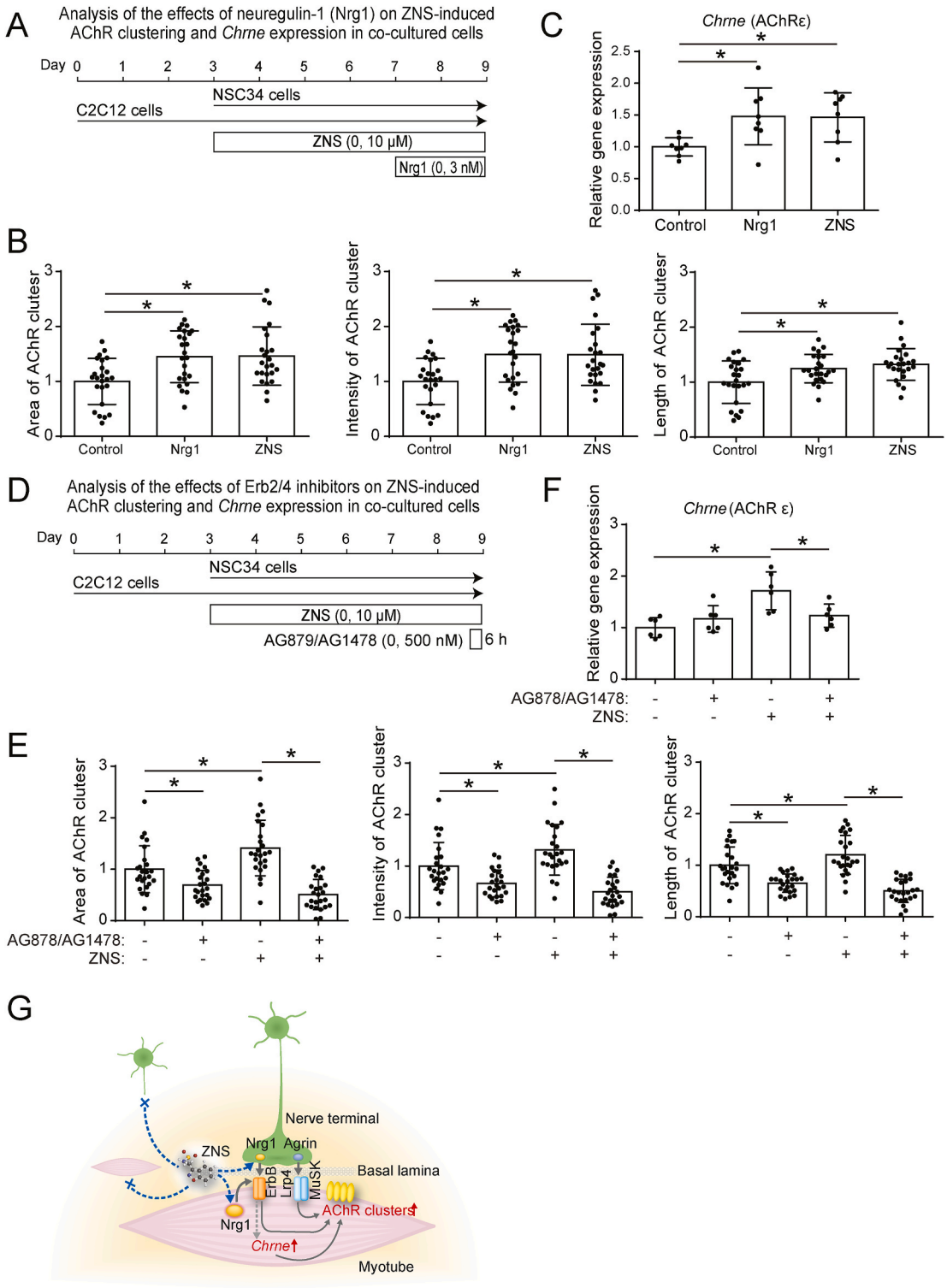

Fig. 5. Nrg1/ErbB signaling are required for ZNS to enhance AChR clustering and *Chrne* expression in co-cultured C2C12 myotubes. **(A, D)** Protocol to co-culture C2C12 myotubes with NSC34 motor neurons with ZNS (0 or 10 μM), Nrg1 (0 or 3 nM), and/or AG879/AG1478 (0 or 500 nM). Two days or 6 h prior to harvesting cells, 3 nM Nrg1 or 500 nM AG879 and 500 nM AG1478, inhibitors of the ErbB receptors, were added to the co-culture medium. **(B, E)** AChR clusters in co-cultured C2C12 myotubes were stained with α-BTX (red). Total area, total intensity, and total length of the AChR clusters were blindly measured using MetaMorph software. Values were normalized to that of co-cultured ZNS-free C2C12 myotubes without Nrg1 **(B)** or AG879/AG1478 **(E)**. Mean and SD are indicated ($n = 24$ optical fields in 4 wells each). **(C, F)** Quantitative RT-PCR to analyze *Chrne* expression in co-cultured C2C12 myotubes. Gene expression was normalized to that of *B2m*, and then to the ratio of ZNS-free cells without Nrg1 **(C)** or AG879/AG1478 **(F)**. Mean and SD are indicated (n = 6 wells each). *$p < 0.05$ by one-way ANOVA followed by Tukey HSD. **(G)** Schematic showing the effect of ZNS on agrin-dependent AChR clustering at the NMJ by activating Nrg1/ErbB signaling. ZNS potentiates agrin-dependent AChR clustering and increases the expressions of NMJ-related genes such as *Chrne* in co-cultured C2C12 myotubes and NSC34 neuronal cells via Nrg1/ErbB signaling. ZNS has no effects on AChR clustering or *Chrne* expression in agrin-treated single cultured C2C12 myotubes, or on Nrg1 expression in single cultured NSC34 motor neurons.

(Fig. 2BC) is unlikely to be accounted for by Rspo2 or GDNF. Other yet unidentified mechanisms may be additionally operational to enhance agrin-dependent AChR clustering by ZNS.

ZNS is prescribed for epilepsy (Brigo et al., 2018) and PD (Murata, 2004). A recent study showed that ZNS increased dopamine production in the dopaminergic neurons by increasing tyrosine hydroxylase in a mouse model of PD that was generated by administration of 1-methyl-4--phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Choudhury et al., 2011). Nrg1 and its receptor, ErbB4, protected dopaminergic neurons in a mouse model of PD (Carlsson et al., 2011). Similarly, administration of Nrg1 increased dopamine levels, and elevated the expression and enzyme activity of tyrosine hydroxylase by activating ErbB4 in the midbrain in mice (Depboylu et al., 2012). These reports indicate that ZNS and Nrg1 have similar effects on the dopaminergic neurons in mouse models of PD. Additionally, Nrg1 is upregulated and acts as an anti-atrophic and anti-apoptotic system in denervated skeletal muscles in mice (Nicolino et al., 2009). Similarly, Nrg1 is involved in the development of skeletal muscle (Kim et al., 1999; Cheret et al., 2013). Nrg1 enhances collateral sprouting of the nerve terminal after partial muscle denervation (Lasiene et al., 2016; Mancuso et al., 2016), and improves motor functions in the $SOD1^{G93A}$ transgenic mice modeling for amyotrophic lateral sclerosis (Modol-Caballero et al., 2020) and a rat model of Charcot-Marie-Tooth disease (Fledrich et al., 2014). If Nrg1 is the identity of an effector of ZNS in our co-cultured cells, the effects of ZNS on PD may also be mediated by Nrg1. Although further pre-clinical and clinical studies are required, ZNS is a promising drug that potentially ameliorates myasthenia gravis, CMS, amyotrophic lateral sclerosis, and Charcot-Marie-Tooth disease. A retrospective study of patients with these NMJ disorders taking ZNS by chance may also be able to disclose the effect of ZNS on the NMJ in clinical settings.

5. Conclusions

We first established a protocol to make *in vitro* NMJs using C2C12 myotubes and NSC34 SMNs. Our protocol was simpler and quicker than those in previous reports. ZNS, an anti-epileptic and anti-Parkinson's disease drug, enhanced the formation of AChR clusters at the *in vitro* NMJs. ZNS upregulated the expression of Nrg1, and inhibition of the Nrg1/ErbB signaling pathways nullified the effect of ZNS. We conclude that ZNS upregulates the expression of Nrg1 at the NMJs, and enhances agrin-dependent AChR clustering.

Disclosure statement

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Declaration of competing interest

The authors declare no competing financial interests.

CRediT authorship contribution statement

Taro Inoue: Methodology, Formal analysis, Writing – original draft. **Bisei Ohkawara:** Methodology, Formal analysis, Writing – original draft. **Samira Bushra:** Methodology, Formal analysis, Resources. **Shunsuke Kanbara:** Methodology, Formal analysis. **Hiroaki Nakashima:** Methodology, Formal analysis, Writing – original draft. **Hiroyuki Koshimizu:** Methodology, Formal analysis. **Hiroyuki Tomita:** Methodology, Formal analysis. **Mikako Ito:** Methodology, Formal analysis. **Akio Masuda:** Methodology, Formal analysis. **Naoki Ishiguro:** Conceptualization, Supervision, Project administration. **Shiro Imagama:** Conceptualization, Supervision, Project administration, Writing – original draft. **Kinji Ohno:** Conceptualization, Supervision, Project administration, Writing – original draft.

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Appendix A. Supplementary data

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