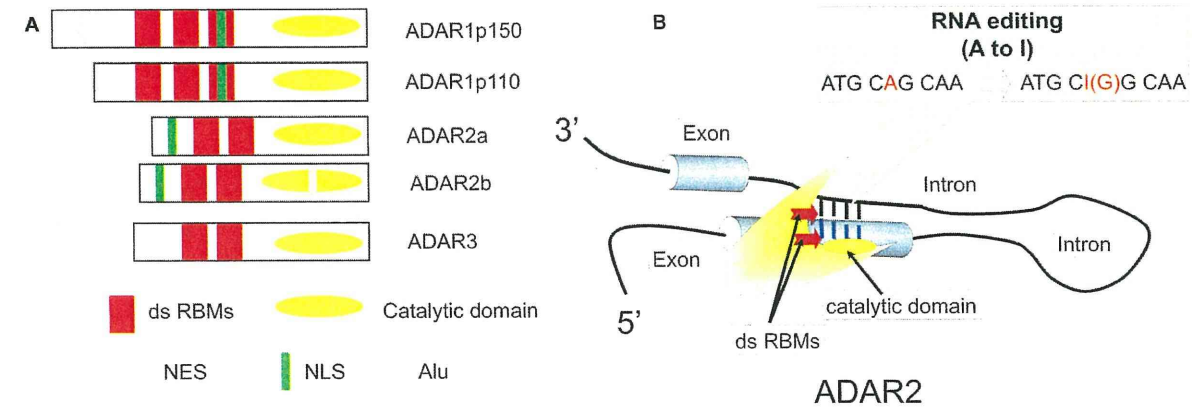


FIGURE 2 | ADAR structure and RNA editing. (A) Three members of the ADAR family. ADAR2 specifically catalyzes the A-to-I conversion at the GluA2 Q/R site, and two isoforms, ADAR2a and more abundantly ADAR2b, are expressed in human brains and spinal cords (Paz et al., 2006). RBM, RNA binding motif; NES, A nuclear export signal; NLS, A nuclear localization signal; Alu, Alu sequences; **(B).** ADAR2 catalyzed adenosine to inosine conversion (A-to-I RNA editing) in the double-stranded RNA structure in both coding and non-coding regions of various transcripts expressed in mammalian brains. When this type of editing occurs in the reading frame, the resulting changes in the amino acid residues alter the biological function of the translated molecules, including 5-HT_{2c} receptors, GluAs, and GluKs. A-to-I RNA editing at the GluA2 Q/R site is specifically catalyzed by ADAR2, a member of the adenosine deaminase acting on RNA (ADAR) family. ADARs act on double-stranded RNA, which is formed with the distal end of exon 11 that includes the coding sequence for the Q/R site and the exon complementary sequence in intron 11 of GluA2 pre-mRNA (Higuchi et al., 1993).

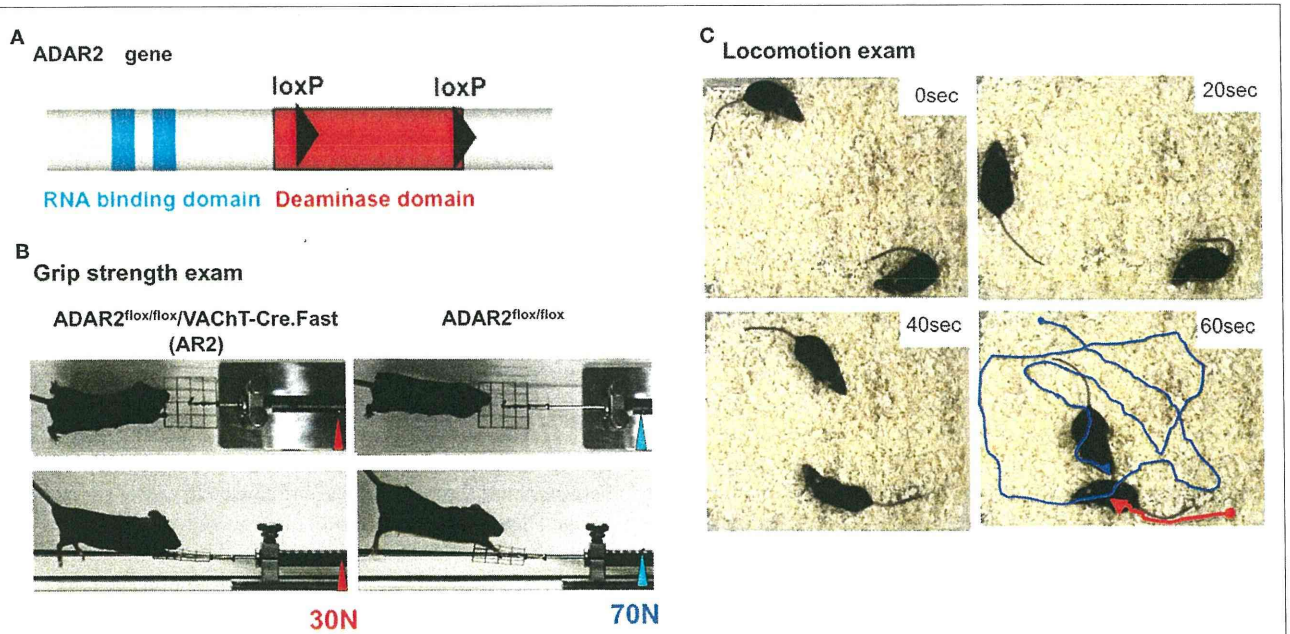


FIGURE 3 | Generation of conditional ADAR2 knockout mice (AR2). (A) One loxP sequence (filled triangle) was inserted into intron 6 and another into intron 9, which covers the majority of the catalytic domain encoded in the ADAR2 gene between the two loxP sites. **(B)** An AR2 mouse at 12 months of age (left panel) exhibits a low grip strength score with an abnormal hind limb and tail posture compared to a control littermate (right panel). **(C)** Locomotion and walking distances for an interval of 1 min were traced using a video capture system. An AR2 mouse at 12 months of age (red line) showed low locomotion levels compared to a control littermate (blue line).

denervated NMJs was higher in AR2 mice at 4 months of age than at 12 months, whereas the proportion of reinnervated NMJs was higher in AR2 mice at 12 months of age than at 4 months of age (**Figure 5D**). These results indicate that degeneration of ADAR2-lacking AHCs led to the degeneration of their axons with a resultant denervation of NMJs, which were reinnervated by collaterally sprouted axons of the ADAR2-expressing normal AHCs. With the degeneration of AHCs, a marked proliferation of glial

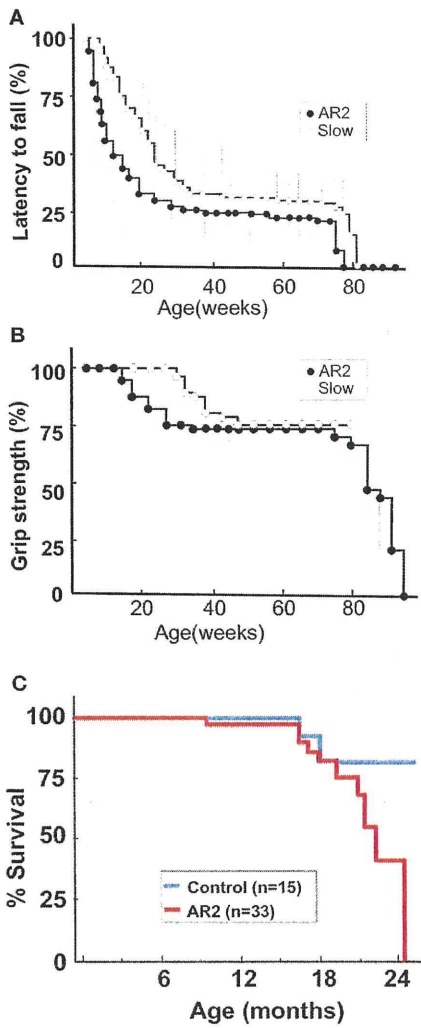
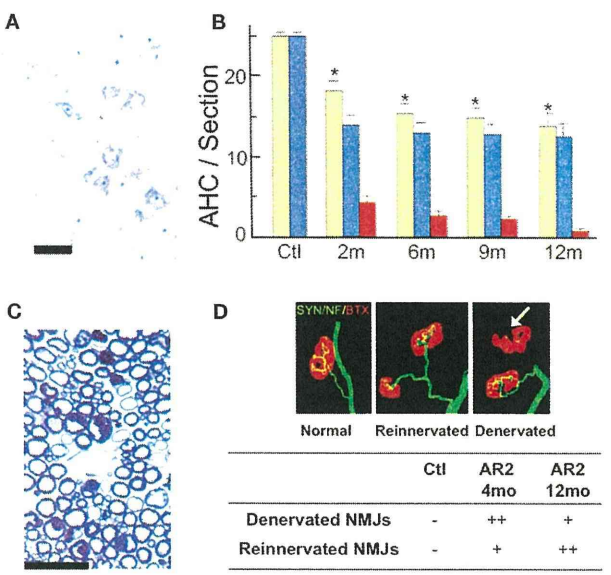


FIGURE 4 | The phenotype of conditional ADAR2 knockout mice (AR2) and slow mice. We generated two lines of conditional ADAR2 knockout mice with different Cre expression patterns: AR2 (ADAR2$^{flox/flox}$/VAChT-Cre.Fast) mice express Cre in progressively larger proportions of motor neurons, reaching ~50% of the maximum by postnatal week 5. Slow (ADAR2$^{flox/flox}$/VAChT-Cre.Slow) mice express Cre more slowly, reaching ~50% of motor neurons by 8 months of age. **(A)** Rotarod performance of AR2 mice (filled circle; $n = 28$) and Slow mice (open circle; $n = 33$). Using a mouse-specific rotarod (SN-445, Neuroscience Corp., Tokyo Japan), the maximal time before falling at 10 rpm during a 180-s period among three trials was used for the analysis. The scores are indicated as a percentage of the performance by control mice ($n = 15$), which exhibited full performance until approximately 12 months of age, followed by a slightly lower performance (greater than 164.5 ± 6.4 s) until 24 months. **(B)** Grip power measured with a dynamometer (NS-TRM-M, Neuroscience, Japan). The score is indicated as a percentage of the performance by control mice. **(C)** AR2 mice had long life spans, but the rate of death increased after month 18 compared to control mice.



	Ctl	AR2 4mo	AR2 12mo
Denervated NMJs	-	++	+
Reinnervated NMJs	-	+	++

FIGURE 5 | Loss of ADAR2-deficient motor neurons. (A) Degenerating large motor neurons in the anterior horn (AHC) of AR2 mice at 2 months of age (Nissl staining). Scale bar, 50 μm. **(B)** The numbers (mean ± SEM) of total AHCs (green columns), ADAR2-positive AHCs (blue columns), and ADAR2-negative AHCs (red columns) in AR2 mice at different ages (2, 6, 9, 12 months). The number of AHCs significantly decreased in AR2 mice after 2 months of age due to the reduction of ADAR2-lacking AHCs ($^*p < 0.01$, repeated ANOVA). **(C)** The ventral root (L5) of AR2 mice at 4 months of age showing degenerating dark axons. Scale bar, 20 μm. **(D)** Neuromuscular junctions (NMJs) and distal motor nerve axons. The quadriceps muscles from an AR2 mouse (Reinnervated, Denervated) at 12 months of age and an age-matched, wild-type mouse (Ctl; normal) are stained with tetramethylrhodamine-bungarotoxin (BTX; red) and immunostained concomitantly with anti-synaptophysin and neurofilament antibodies (SYN/NF; green). The endplates (red) were counted as "innervated" if they were merged with axon terminals (yellow). A thick axon terminal in the Ctl mouse innervates each endplate, whereas in AR2 mice, some NMJs are innervated by axons that simultaneously innervate more than one NMJ (reinnervated NMJ), and other NMJs are devoid of axon terminals (denervated NMJs; white arrow).

fibrillary acidic protein (GFAP)-positive astrocytes and MAC2-positive microglial cells was detected in the anterior horns of AR2 mice (**Figures 6A,B**).

Other than the motor neurons in the spinal cord, large neurons in the facial (VII), and hypoglossal nerve nuclei (XII) in AR2 mice at 12 months of age were significantly decreased in number. In contrast, the number of neurons in the nuclei of the extraocular motor nerves (III, IV, VI) was not decreased (**Figure 6C**). Conversely, GluA2 Q/R site editing was significantly decreased in the oculomotor nerve nuclei (89.7% of control mice, $p = 0.0048$) and the facial nerve nuclei (83.3% of control mice, $p = 0.0017$) of AR2 mice at 12 months of age (**Figure 6C**). These results indicate that subsets of motor neurons, including those in the oculomotor nerve nucleus, are relatively resistant to cell death mediated by deficient ADAR2. Notably, the selective sparing of motor neurons that innervate the extraocular muscles as compared to those that innervate the bulbar and limb muscles is characteristically seen in ALS patients. Motor neurons in the nuclei of the oculomotor nerves are much less vulnerable in ALS patients. Notably, the expression of Ca^{2+}-binding proteins, particularly parvalbumin, is high in oculomotor neurons and low in the facial and spinal motor neurons (

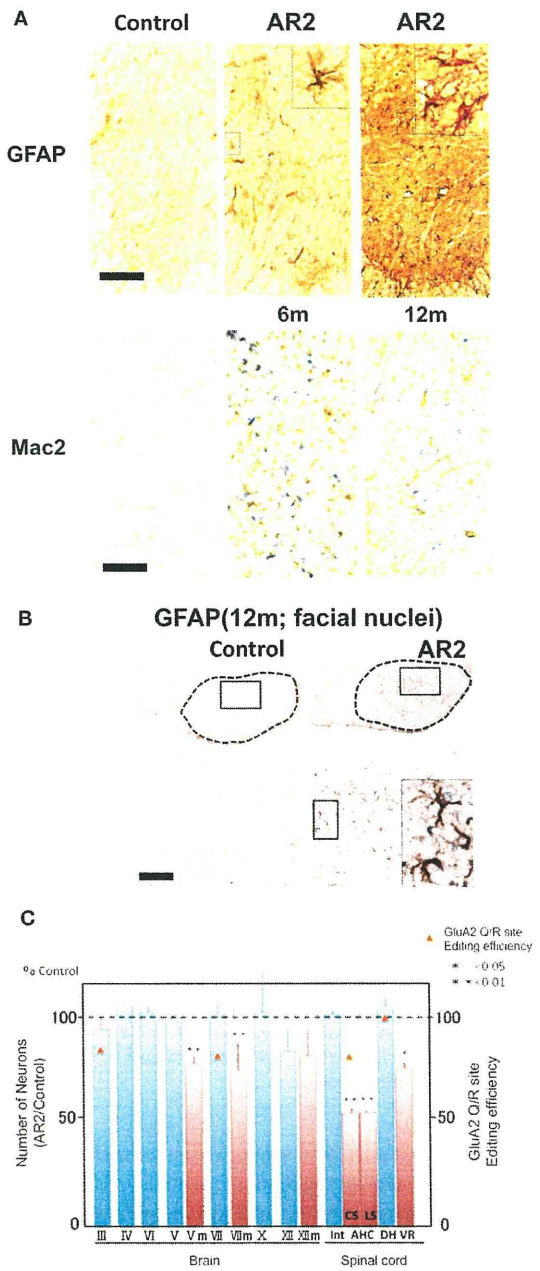


FIGURE 6 | Reactive glial cells in the spinal anterior horns and cranial motor nerve nuclei of AR2 mice (A,B). Immunohistochemistry in the anterior horn (C5) and motor trigeminal nuclei (Vm). There was an increase in GFAP- and MAC2-immunoreactivity in the spinal anterior horn of the AR2 mice, indicating the proliferation of activated astrocytes and microglia in association with motor neuron degeneration. m: months of age, inset; activated astroglia. Scale bars, 100 μm (GFAP) and 50 μm (insets and Mac2). **(C)** In AR2 mice at 12 months of age, the number of large neurons in the cranial nuclei of Vm, VII, and XII were lower than those in age-matched control mice, whereas those in the cranial nuclei III, IV, and VI were not. The GluA2 Q/R site editing efficiency was decreased in III, VII, and AH, indicating that the cranial motor neurons that innervate extraocular muscles are exceptionally resistant to deficient-ADAR2-mediated neuronal death.

1993). It has been shown that over-expression of parvalbumin attenuated kainate-induced Ca^{2+} transients and protected spinal motor neurons from the resultant neurotoxicity in parvalbumin transgenic mice (Van Den Bosch et al., 2002). In AR2 mice and in patients with sporadic ALS, it is likely that neurons with abundant parvalbumin, such as ocular motor neurons, are more resistant to Ca^{2+} overload from Ca^{2+}-permeable AMPA receptors than those with low parvalbumin levels, such as spinal motor neurons.

THE CRUCIAL ROLE OF GluA2 RNA EDITING IN THE DEATH OF ADAR2-LACKING MOTOR NEURONS IN AR2 MICE

ADAR2 predominantly catalyzes the RNA editing at the Q/R site of GluA2 both *in vivo* and *in vitro* (Melcher et al., 1996a; Higuchi et al., 2000; Wang et al., 2000), but there are numerous A-to-I positions in mammalian brains, some of which are specifically catalyzed by ADAR2. Among the A-to-I positions specifically mediated by ADAR2 (Nishikura et al., 2008), we found a significant reduction in the editing efficiency at the GluK2 (GluR6) Q/R site (AR2 mice vs. control mice, 15.3 vs. 31.8%, $p = 0.04416$) and at the K/E site of cytoplasmic fragile X mental retardation interacting protein 2 (CYFIP2) mRNA (**Figure 7**). These results indicate that RNA editing at the ADAR2-mediated A-to-I positions is universally defective in AR2 motor neurons.

To examine the possible role of defective RNA editing at A-to-I positions other than the GluA2 Q/R site in motor neuron death, we investigated the effects of edited GluA2 expression in ADAR2-lacking motor neurons (**Figure 8**). We exchanged the endogenous *GluA2* alleles that encoded Q at the Q/R site in AR2 mice with the GluR-B^R alleles (Kask et al., 1998), which encode R at the Q/R site of GluA2. This exchange circumvented the need for ADAR2-mediated RNA editing in the expression of edited GluA2. We intercrossed *ADAR2$^{flox/+}$*/VAChT-Cre.Fast/GluR-B$^{R/+}$ mice to generate AR2/GluR-B$^{R/R}$ mice. AR2/GluR-B$^{R/R}$ mice (AR2res) were phenotypically normal and had full motor function until

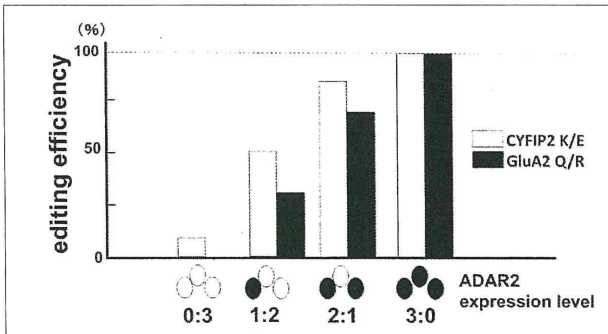


FIGURE 7 | The failure of CYFIP2 K/E site editing and GluA2 Q/R site editing in ADAR2-lacking motor neurons. When the extent of RNA editing was measured in the pooled lysates of three motor neurons obtained from AR2 mice, the proportions of CYFIP2 mRNA edited at the lysine/glutamic acid (K/E) site and those of Q/R site-edited GluA2 mRNA were lower in the lysates containing ADAR2-lacking motor neurons. This finding indicates that ADAR2 specifically mediates A-to-I conversion at the K/E site of CYFIP2 pre-mRNA. Ratios in abscissa indicate the number of ADAR2-expressing motor neurons (filled circle) and ADAR2-lacking motor neurons (open circle) in the three motor neuron lysates of AR2 mice. (see **Figure 10**).

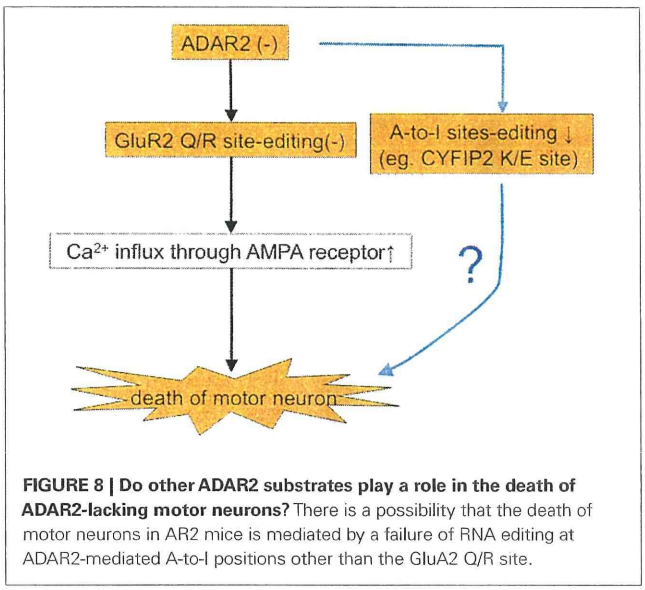


FIGURE 8 | Do other ADAR2 substrates play a role in the death of ADAR2-lacking motor neurons? There is a possibility that the death of motor neurons in AR2 mice is mediated by a failure of RNA editing at ADAR2-mediated A-to-I positions other than the GluA2 Q/R site.

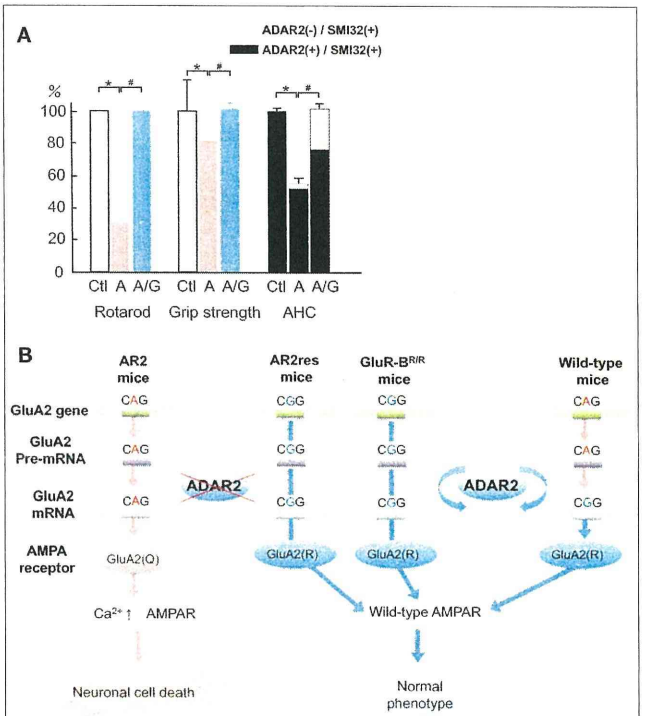


FIGURE 9 | The crucial role of GluA2 Q/R site editing in the death of ADAR2-deficient motor neurons. (A) AR2/GluR-$B^{ti/ti}$ mice (AR2res) are phenotypically normal with a preservation of ADAR2-lacking AHCs that account for 30% of the total AHCs at 6 months of age. **(B)** A scheme illustrating that the death of ADAR2-lacking motor neurons due to failure to edit the GluA2 Q/R site in the AR2 mice is rescued in the AR2res mice. The exonic Q codon (CAG) for the Q/R site of GluA2 is substituted by an R codon (CGG) in the endogenous *GluA2* alleles, and only edited GluA2 is expressed in the absence of ADAR2 activity.

6 months of age. The AHCs, including those lacking ADAR2 due to Cre-mediated recombination, were viable in AR2res mice at 6 months of age, and the total number of AHCs was the same as in age-matched control mice (**Figure 9A**). Consistent with a lack of AHC loss, there was no detectable increase in GFAP- or MAC2-immunoreactivity in the anterior horns (Hideyama et al., 2010). These results demonstrate that an ADAR2 deficiency induces the slow death of motor neurons specifically via the GluA2 Q/R site editing failure (**Figure 9B**).

DEATH OF MOTOR NEURONS IN HETEROZYGOUS AR2 (HETEROAR2) MICE

The results of our experiments with the AR2 mice indicate that deficient-ADAR2-mediated death of motor neurons in the spinal cord and the cranial motor nerve nuclei is specifically mediated by the failure to edit the GluA2 Q/R site. In the ALS spinal cord, some motor neurons express unedited GluA2, but others express only edited GluA2. The majority of motor neurons expressing unedited GluA2 also express edited GluA2 (Kawahara et al., 2004). Furthermore, a recent immunohistochemical study demonstrated that both ADAR2-positive and ADAR2-negative motor neurons coexist in patients with sporadic ALS, whereas all motor neurons are ADAR2-positive in control subjects (Aizawa et al., 2010). However, we do not know the expression level of ADAR2 that is required to edit all GluA2 mRNA or the proportion of unedited GluA2 that is not harmful to motor neurons.

To answer these questions, we investigated the extent of GluA2 Q/R site editing in motor neurons lacking one ADAR2 allele in the heterozygous $ADAR2^{flox/+}$/VAChT-Cre (HeteroAR2) mice. Additionally, we investigated whether motor neurons lacking one ADAR2 allele can survive in HeteroAR2 mice compared to AR2 and control mice. In HeteroAR2 mice, the proportion of motor neurons that express Cre is the same as in AR2 mice; the Cre-expressing motor neurons express only one ADAR2 allele. Therefore, if the expression level of ADAR2 in normal motor neurons is sufficiently above the requirement to edit the Q/R site of all GluA2 mRNAs expressed (i.e., threefold or more), all motor neurons would express only edited GluA2. However, if normal motor neurons express ADAR2 at a level that is only sufficient to edit GluA2 (i.e., less than twofold), motor neurons with one ADAR2 allele would express abundant unedited GluA2 and die. Furthermore, if motor neurons expressing one ADAR2 allele express both edited and unedited GluA2 and undergo degeneration in HeteroAR2 mice, we would expect to find the proportion of unedited GluA2 that is toxic to motor neurons.

When the extent of GluA2 Q/R site editing was examined in the lysates of three laser-captured motor neurons of 2-month-old HeteroAR2 mice, GluA2 Q/R site editing was incomplete in approximately 20% of the lysates (**Figure 10**). The proportion of edited GluA2 was above 70% in all the lysates; however, in the AR2 mice of the same age, unedited GluA2 was detected in more than 60% of the lysates of three motor neurons, and the editing efficiency was 0 in 7% of the lysates examined (Hideyama et al., 2010). Therefore, it is likely that motor neurons exhibit considerable editing activity in the expression of only one ADAR2 allele; however, this is not sufficient to edit the Q/R site of all GluA2 mRNA in HeteroAR2 mice.

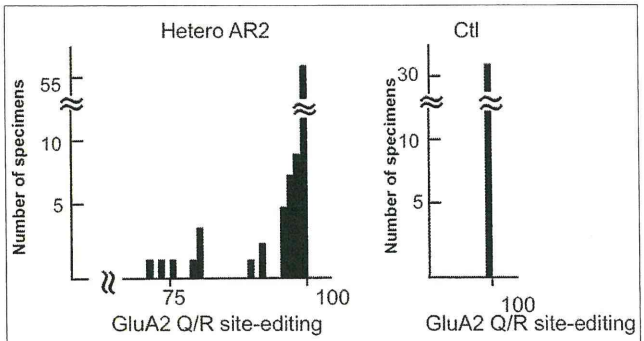


FIGURE 10 | Some of the motor neurons from HeteroAR2 mice express Q/R site-unedited GluA2 mRNA. A frequency histogram of editing efficiency at the GluA2 Q/R site in the lysates of three motor neurons obtained from heterozygous ADAR2$^{+/-}$/VAChT-Cre.Fast (HeteroAR2) mice and control mice (Ctl). Motor neurons (252 neurons) were dissected from 14-μm-thick frozen sections of spinal cord obtained with a laser microdissector from HeteroAR2 mice at 2 months of age ($n = 4$). The lysates containing three motor neurons (84 specimens) were subjected to a measurement of the editing efficiency of GluA2 mRNA at the Q/R site. This histogram shows that approximately a quarter of the lysates, or 15–20% of AHCs, express unedited GluA2. Notably, the extent of GluA2 editing is above 70%, which is in contrast with the AR2 mice, in which approximately 8% of the lysates expressed only unedited GluA2. All 96 motor neurons dissected from control mice (Ctl; ADAR2$^{flox/flox}$ and VAChT-Cre mice) expressed only Q/R site-edited GluA2 mRNA.

We also examined whether there was a loss of AHCs in HeteroAR2 mice. The extent of AHC loss in HeteroAR2 mice was approximately half (26%) of that observed in AR2 mice (46%) at 12 months of age (**Figure 11A**). A moderate increase in GFAP- and MAC2-immunoreactivity was detected in the anterior horns of HeteroAR2 mice at 12 months of age (**Figure 11B**). HeteroAR2 mice did not exhibit significant behavioral changes until 12 months of age, indicating that mild loss of motor neurons would not affect motor function at least until 1 year of age.

These results show that one ADAR2 gene allele is sufficient to edit all GluA2 mRNA in half of the motor neurons but is insufficient in the other half of motor neurons. Because the editing efficiency was above 70% in the motor neuron lysates of HeteroAR2 mice, it is likely that the minimal expression level of ADAR2 required for complete GluA2 editing is slightly higher than half of the normal level in mouse motor neurons.

More than a quarter of the motor neurons in HeteroAR2 mice underwent degeneration by the age of 1 year. Furthermore, from the results of our experiments with AR2 and AR2res (Hideyama et al., 2010), it is likely that only the motor neurons expressing unedited GluA2 have undergone degeneration. The proportions of unedited GluA2 are 30% at the maximum and less than 10% in the majority of motor neurons in HeteroAR2 mice, indicating that expression of unedited GluA2, even in a small proportion, is not favorable for the survival of motor neurons in mice.

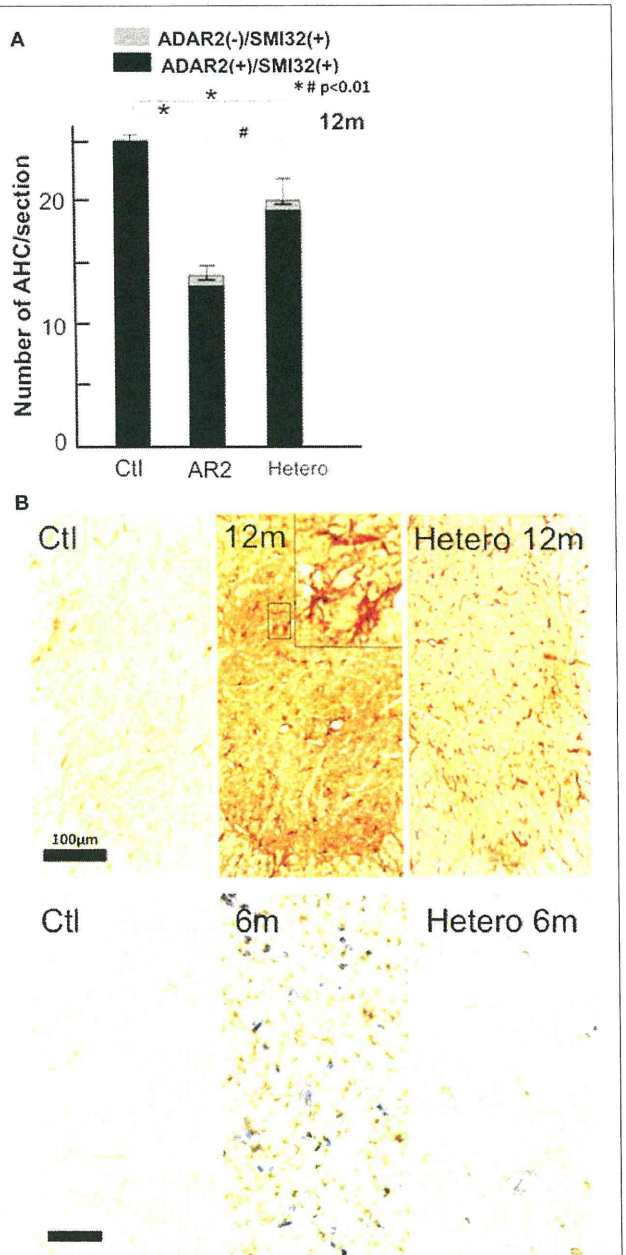


FIGURE 11 | HeteroAR2 mice display a loss of AHCs. (A) The numbers (mean ± SEM) of ADAR2-positive AHCs (black columns) and those of ADAR2-lacking AHCs (gray columns) in HeteroAR2 mice at 12 months of age compared to age-matched AR2 and control mice. There was a loss of AHCs in the HeteroAR2 mice (Hetero; $^{*}p < 0.01$) but to a lesser extent than in the AR2 mice ($^{\#}p < 0.01$). **(B)** Immunohistochemistry in the anterior horn of the spinal cord (C5). There was an increase in GFAP-immunoreactivity but to a lesser extent than in the AR2 mice (upper panel) and a slight increase of MAC2-immunoreactivity (lower panel) in the HeteroAR2 mice. Scale bars: 100 μm (upper panel) and 50 μm (insets and lower panel).

THE ADAR2 HYPOTHESIS FOR SPORADIC ALS

Progression of ALS is rather slow, taking several years to progress from the onset of the initial symptoms to death, which results from failure of the respiratory muscles. From our findings in the AR2 mice, we learned that some ADAR2-lacking motor neurons died within 1 month of ADAR2 knockout, whereas others could survive more than 1 year even with only unedited GluA2

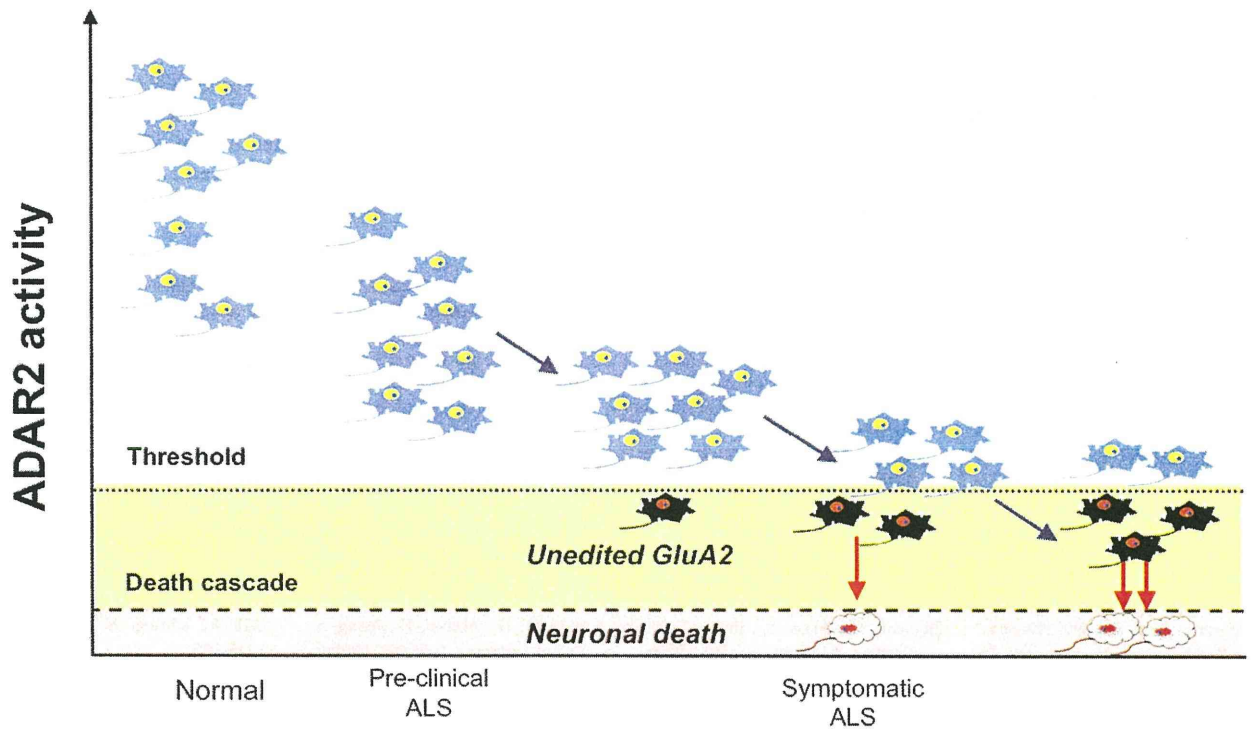


FIGURE 12 | The ADAR2-GluA2 hypothesis for ALS pathogenesis. ADAR2 activity is preserved well above the level that enables motor neurons to edit the Q/R site of all GluA2 mRNA expressed in healthy individuals (normal; neurons in blue). In the motor neurons of sporadic ALS patients, ADAR2 activity progressively decreases, and the ADAR2 activity in all motor neurons is initially low but is within a level that is sufficient to edit the Q/R site of all GluA2 mRNA; thus, motor neurons function normally (pre-clinical ALS). With a decline of ADAR2 activity, some motor neurons express ADAR2 below a threshold level for GluA2 RNA editing (dotted line) and express unedited GluA2 (neurons in black). These motor neurons can survive for some period of time, but they do not function normally and ultimately enter into the death cascade in a stochastic manner. With the progressive decline of ADAR2 activity, the number of motor neurons expressing unedited GluA2, and therefore those destined to die, increases. Patients and neurologists recognize ALS only after a considerable proportion of motor neurons has already malfunctioned (neurons in black) or been lost (neurons in gray).

expression (Hideyama et al., 2010). Therefore, it is likely that the timing of motor neuron death may be a stochastic phenomenon that depends on environmental factors and the level of the compensatory activity in the individual neurons, including the firing frequency of the motor neurons, the strength of the Ca^{2+} buffering system, and the density of functional Ca^{2+}-permeable AMPA receptors. Furthermore, the results from the HeteroAR2 mice indicate that motor neurons expressing unedited GluA2, regardless of the proportion, are destined to die in sporadic ALS patients.

The progressive downregulation of ADAR2 activity increases the number of motor neurons expressing unedited GluA2 in sporadic ALS. The mechanism underlying the reduction of ADAR2 activity in ALS motor neurons is not clear; however, considering that inefficient GluA2 RNA editing was found only in sporadic cases (Kawahara et al., 2004), undefined postnatal factors regulating the ADAR2 activity should not be neglected. Because ADAR2 SNPs are associated with longevity syndrome (Sebastiani et al., 2009) and the age-dependent downregulation of ADAR2 activity has been shown in human brains (Nicholas et al., 2011), the acceleration of age-related neuronal dysfunction may have a role in the progressive reduction of ADAR2 activity in ALS motor neurons.

We recently reported that TDP-43 pathology, which is a hallmark of ALS, appeared only in the motor neurons lacking ADAR2 immunoreactivity in patients with sporadic ALS (Aizawa et al., 2010). It is likely that motor neurons lacking ADAR2 immunoreactivity represent those expressing unedited GluA2, and motor neurons expressing ADAR2 immunoreactivity with normal TDP-43 immunoreactivity represent those expressing only edited GluA2. Because a reduction of ADAR2 likely begins before motor neurons express unedited GluA2 in ALS motor neurons, TDP-43 pathology may be induced by the expression of unedited GluA2 rather than TDP-43 pathology causes reduced ADAR2 activity. We do not know whether the reduction of ADAR2 immunoreactivity in the ALS motor neurons results from a reduced gene expression or accelerated ADAR2 protein degradation catalyzed by Ca^{2+}-activated proteinase as demonstrated in ischemic rat brains (Muhich et al., 2011).

Based on this evidence, we propose a hypothesis for the pathogenesis of sporadic ALS. ALS motor neurons express progressively lower ADAR2 activity before manifesting an ALS phenotype, and the pathological process commences when motor neurons begin to express unedited GluA2. Motor neurons expressing unedited GluA2 do not function normally but do not immediately die.

The timing of the entry of these motor neurons into the death cascade may be regulated in a stochastic manner. With a sequential progression of these events, the pool of normally functioning motor neurons expressing only edited GluA2 decreases, which ultimately induces the ALS phenotype in patients (**Figure 12**). Because mutations in the coding molecules of the genes involved in RNA regulation, including TDP-43 and FUS/TLS, were recently found in patients with familial ALS (Arai et al., 2006; Neumann et al., 2006; Kwiatkowski et al., 2009; Vance et al., 2009), dysregulation of RNA metabolism in ALS pathogenesis is now attracting the interest of researchers (Lemmens et al., 2010). Because ADAR2 is an RNA regulatory molecule, future studies are needed to elucidate the molecular link between abnormalities of these ALS-linked RNA regulatory molecules with the ADAR2 downregulation in ALS motor neurons. Forcing motor neurons to express only edited GluA2 may be a future therapy for sporadic ALS.

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Research Article

Traditional Chinese Medicine Improves Activities of Daily Living in Parkinson's Disease

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We evaluated the effects of a traditional Chinese medicine (TCM), named Zeng-xiao An-shen Zhi-chan 2 (ZAZ2), on patients with Parkinson's disease (PD). Among 115 patients with idiopathic PD enrolled (mean age, 64.7 ± 10.2 years old), 110 patients (M = 65, F = 45; mean age, 64.9 ± 10.7 years old) completed the study. Patients took either ZAZ2 ($n = 59$) or placebo granule ($n = 56$) in a blind manner for 13 weeks while maintaining other anti-Parkinson medications unchanged. All participants wore a motion logger, and we analyzed the power-law temporal autocorrelation of the motion logger records taken on 3 occasions (before, one week, and 13 weeks after the drug administration). Drug efficacy was evaluated with the conventional Unified Parkinson Disease Rating Scale (UPDRS), as well as the power-law exponent α, which corresponds to the level of physical activity of the patients. ZAZ2 but not placebo granule improved the awake-sleep rhythm, the UPDRS Part II, Part II + III, and Part IV scores, and the α values. The results indicate that ZAZ2 improved activities of daily living (ADL) of parkinsonism and, thus, is a potentially suitable drug for long-term use.

1. Introduction

Conventional anti-parkinsonism drugs effectively ameliorate the symptoms of patients with Parkinson's disease (PD) during the initial several years of onset, but become increasingly less effective and induce motor fluctuations including wearing-off, on-off, dopa-induced dyskinesia, and agonist-induced sleep attack [1–5]. PD patients not infrequently suffer from nonmotor symptoms, such as neuropsychiatric symptoms, autonomic symptoms, gastrointestinal symptoms, sensory symptoms, nonmotor fluctuations (autonomic symptoms, cognitive or psychiatric symptoms, sensory symptoms including pain), fatigue, and sleep disturbance [6–8], and these nonmotor symptoms may be intrinsic to the disease pathology or may be the result of treatment with dopaminergic agents. Several studies have established that the nonmotor symptoms of PD are common, occur across all stages of PD, and are a key determinant of quality of life [7].

Herbal remedies have a long history of use (particularly in East Asian countries) for alleviating various symptoms and have been increasingly used as alternative medicines worldwide, including the United States [9]. Traditional Chinese medicines (TCMs) ameliorate various symptoms, particularly the ageing-related symptoms [10, 11], and hence are likely to be beneficial for chronic diseases such as PD [12–14]. Good compliance for long-term use with few side effects may be another merit of TCM suitable for patients with PD [12–14].

In order to evaluate the effects of TCM on symptoms of parkinsonism, we used Zeng-xiao An-shen Zhi-chan 2 (ZAZ2) in this study. In addition, we adopted a recently developed method analyzing the power-law temporal autocorrelation of wrist activity measured with a motion logger

[15–17] in order to evaluate the efficacy of ZAZ2 on parkinsonism. Pan et al. showed that the power-law exponent (α) for higher levels or at the so-called local maxima of coefficients of the wavelet transform significantly and in correlation with the symptom severity of PD patients [15]; hence analysis of α is likely to be useful for evaluating the effects of TCM on parkinsonism as a whole. The aim of the present study was to evaluate the ameliorating effects of ZAZ2 on impaired motor and nonmotor symptoms of PD patients using the UPDRS scores, secondary symptoms scores [18], and also by analyzing the mean activity levels and the power-law temporal exponents of *MicroMini-Motionlogger*, Ambulatory Monitoring Inc. (AMI) scores.

2. Methods

2.1. Subjects. Of the 140 PD patients who visited the clinic at the Department of Neurology of Shuguang Hospital Affiliated to Shanghai University of TCM between July 2008 and April 2010, 115 patients with idiopathic PD (mean age ± SD, 64.7 ± 10.2 years old, mean duration of illness, 5.5 ± 7.3 years) who fulfilled the inclusion criteria, were invited to participate in the study. The UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria were used [19]. PD was defined by the presence of at least two of the four cardinal features (bradykinesia, tremor, rigidity, and postural reflex abnormality). Other forms of parkinsonism based on laboratory tests such as MRI were excluded. All the patients were at least 40 years of age and were evaluated in the middle of their levodopa dose cycle at maximal mobility (on) for the severity of parkinsonism, and signed informed consent before participation. Next, the patients were double blindly grouped into the TCM group (n = 59, 64.27 ± 11.8) or the placebo group (n = 56, 63.91 ± 13.9) (Table 1). Patients were randomly assigned to the ZAZ2 or placebo group and given random numbers by a study coordinator, who also encoded the drugs with matching random numbers. Neither the patients nor the researchers monitoring the outcome knew which patient was receiving which treatment, until the study was over and the random code was broken. Anti-parkinsonism drug administration was not changed throughout the experiment. The study was approved by The Ethics Committee of Shuguang Hospital Affiliated to Shanghai University of TCM, and performed under the principles outlined in the Declaration of Helsinki; all subjects provided informed consent in accordance with institutional requirements prior to participation in the study.

2.2. Additional Treatment. Zeng-xiao An-shen Zhi-chan 2 (ZAZ2), the TCM used in this study, is a granule made up of 14 kinds of herbs: *Uncaria rhynchophylla* 10 g, *Rehmanniae radix* 10 g, *Cornus officinalis* 8 g, *Asnaragus cochinchinensic* 10 g, *Paeonia lactiflora* 10 g, *Desertliving cistanche* 10 g, *Puerariae radix* 10 g, *Arisaema consanguineum Schott* 10 g, *Salviae Miltiorrhizae radix* 10 g, *Acorus tatarinowii* 10 g, *Curcuma longa Linn* 12 g, *Morindae officinalis radix* 10 g, *Rhizoma gastrodiae* 10 g, and *Rhizoma chuanxiong* 10 g. ZAZ2 is commonly used in "insufficiency of Kidney yang" in China. Placebo granules were made up of 5 kinds of herbs: *Largehead atractylodes rhizome* 10 g, *Poria cocos (Schw.) wolf* 10 g, *Jobstears seed* 10 g, *Malt* 10 g, and *Chinese date* 10 g. These 5 herbs have no activity in terms of traditional Chinese medicine [13]. Patients were instructed to take one package (8 g) of ZAZ2 or placebo soluble granule three times a day at least 30 min before or after the ingestion of other drugs for three consecutive months (13 weeks). The shape and color of ZAZ2 and the placebo soluble granule are very alike and cannot be distinguished from one another by appearance or aqueous solution taste. ZAZ2 and the placebo granule were made by the manufacturing laboratory of Shuguang Hospital Affiliated to Shanghai University of TCM. The trial was carried out as a randomized, double-blind, parallel group study.

Table 1: Patient characteristics.

	Placebo (n = 54)	ZAZ2 (n = 56)
Age (yr)	63.1 ± 10.2	62.82 ± 10.31
Men/Women	32/22	34/22
Disease duration (yr)	5.81 ± 3.24	5.73 ± 4.81
Hoehn & Yahr stages	2.35 ± 1.33	2.37 ± 1.13
Levodopa/DCI (mg/day)	396.61 ± 159.24	390.59 ± 164.71
Pramipexole (mg/day)	1.03 ± 0.69	1.00 ± 0.79
Selegiline Hydrochloride (mg/day)	8.97 ± 5.66	9.28 ± 4.95

DCI: decarboxylase inhibitor.

2.3. Equipment. All patients wore a small watch-type activity monitor equipped with a computer (*MicroMini-Motionlogger*, Ambulatory Monitoring, Inc, Ardsley, New York) on the wrist of their nondominant hand for seven consecutive days before taking test granule (week 0), one week (week 1), and 13 weeks (week 13) after taking test granule. Zero-crossing counts were recorded every one minute to register and quantify human physical activity [15], and the data was stored in internal memory. After recording, data were transmitted to an external computer by software installed on the device.

2.4. Assessments. Daily profiles and mean counts: We plotted the activity scores for 7 consecutive days to see the daily profiles and biological rhythms of each patient (Figure 1). The records acquired during awake times and sleep times were separated with Action-W, Version 2 (Ambulatory Monitors Inc., Ardsley, NY, USA). The mean counts during awake times and sleep times were separately calculated for each record (Table 2 and Figure 2(a)).

UPDRS Scores. The UPDRS of all patients were evaluated at week 0, week 1, and week 13 by neurologists who were blinded to the test granule.

Secondary Symptom Score [18]. This score is conventionally used in China to evaluate the effects of anti-parkinsonism drugs and consists of 8 parts, including the assessments of nonfluent speech, vertigo, insomnia/nightmares, headache,

Table 2: Results of clinical evaluation between before and after test granule administration.

	Placebo ($n = 54$)			ZAZ ($n = 56$)		
	Week 0	Week 1	Week 13	Week 0	Week 1	Week 13
UPDRS total score	46.6 ± 16.3	44.7 ± 15.3	45.9 ± 18.1	46.3 ± 17.1	37.1 ± 11.2$^{*\#\#}$	40.7 ± 15.1$^{*\#}$
UPDRS I	2.5 ± 0.7	2.3 ± 1.1	2.4 ± 1.2	2.6 ± 0.8	2.1 ± 0.7*	2.3 ± 0.9
UPDRS II	15.7 ± 9.3	14.8 ± 11.2	15.3 ± 11.6	15.9 ± 11.3	12.5 ± 4.6$^{*\#}$	13.4 ± 9.8$^{*\#}$
UPDRS III	25.5 ± 12.9	23.8 ± 10.6*	24.9 ± 12.7	25.4 ± 10.1	19.3 ± 9.8$^{*\#}$	21.6 ± 10.4*
UPDRS IV	3.1 ± 1.1	2.9 ± 1.6	3.0 ± 1.4	3.2 ± 1.4	2.6 ± 0.8$^{*\#}$	2.7 ± 1.3$^{*\#}$
Awake time (counts/min)	98.5 ± 14.1	102.6 ± 18.9	100.7 ± 16.9	99.8 ± 17.8	126.7 ± 13.4$^{*\#\#}$	118.4 ± 11.8$^{*\#\#}$
Sleep time (counts/min)	42.9 ± 17.1	38.8 ± 15.6*	40.1 ± 14.8	43.2 ± 11.6	35.6 ± 13.6$^{*\#}$	32.8 ± 13.6$^{*\#}$
α (awake time)	0.97 ± 0.21	0.95 ± 0.28	0.96 ± 0.18	0.97 ± 0.24	0.88 ± 0.21$^{*\#}$	0.86 ± 0.19$^{*\#\#}$
α (sleep-time)	1.19 ± 0.28	1.16 ± 0.27	1.15 ± 0.29	1.18 ± 0.26	1.04 ± 0.22$^{*\#}$	1.02 ± 0.18$^{*\#\#}$

Data presented are mean ± SD. $^{*}P < .05$; $^{**}P < .01$ compared to week 0 (repeated-measure ANOVAs); $^{\#}P < .05$; $^{\#\#}P < .01$ compared to placebo (Bonferroni test); UPDRS: Unified Parkinson's Disease Rating Scale; α: power-law exponent.

sweating or night sweats, tiredness, sense of cold, and dysuria. In this study, the secondary symptom scores were evaluated in week 0, week 1, and week 13 for all participants by the same neurologists, and it reflects the opinion of PD patients (Table 3).

Power-Law Temporal Analysis α. The methods for power-law temporal analyses were the same as those described in Pan et al. [15]. The awake time and sleep time data were used separately for the power-law temporal analyses. After integrating the time series, the data were wavelet-transformed using the third derivative of the Gaussian function as the so-called "mother wavelet." The wavelet coefficients (W (S)) at each point along the time series and at different timescales (S) were obtained by convolving the mother wavelet with the time series. This approach facilitates the probing of transient increases or decreases in detrended activity records at different timescales. The transient increases (low-high-low level activity patterns) yielded local maxima of the wavelet coefficients at their time points, while the decreases (high-low-high level activity patterns) yielded local minima of the wavelet coefficients. Next, the squared wavelet coefficients at the local maxima or minima were averaged for all the data points, and the power-law exponent (α) was obtained separately for local maxima and minima as the slope of a straight line fitted in the double-logarithmic plot of S versus W $(S)^2$ in the range of S corresponding to 8 to 35 min. The power-law exponent of maxima has been successfully used for the assessment for PD in previous studies [15, 20]. In this study, we analyzed the local maxima separately for the awake time and sleep time of each record (Table 2 and Figure 2(b)).

For the safety assessments, each patient underwent a physical examination by a physician and laboratory tests for blood counts and biochemistry, and urinalysis at each visit.

2.5. Statistical Analysis. Repeated-measure ANOVAs were conducted to test the differences among week 0, week 1, and week 13 in the ZAZ2 and placebo groups. When a significant difference was detected, a post-hoc test (Bonferroni test) was conducted between the ZAZ2 and placebo groups compared for the UPDRS total score, UPDRS Part I, Part II, Part III, Part II + Part III, and Part IV, mean values, and the power-law temporal α in awake time and sleep time. A significant difference was defined as $P < .05$. SPSS windows Version 17.0 was used for statistical analyses. All data are expressed as the mean ± standard deviation.

3. Results

Five patients dropped out of the study; one patient in the ZAZ2 group was unable to tolerate the bitter taste of ZAZ2, while two in the ZAZ2 group and two in the placebo group dropped out due to a conflict with other TCM prescribed for concomitant diseases. Neither physical examination nor laboratory tests revealed any adverse changes after additional treatment in either group.

The post-hoc test revealed no significant differences in baseline (week 0) UPDRS scores, Hoehn & Yahr stages, mean counts, and power-law temporal exponent α values, or in the dosage of L-dopa/DCI, Dopamine agonist or monoamine oxidase B, between the ZAZ2 and placebo groups (Tables 1 and 2).

Daily profiles of AMI counts clearly demonstrated improvement of the biological rhythm after the additional treatment in the ZAZ2 group (Figure 1(a)) but not in the placebo group (Figure 1(b)). Patients in the ZAZ2 group showed a more frequent switch from high activity to low activity in awake time and lower activity during sleep time after ZAZ2 than before ZAZ2 (Figures 1(a) and 2(a), Table 2, $P < .05$, Bonferroni test). Such changes were not observed after placebo granule intake (Figures 1(b) and 2(a), Table 2).

When the effects of ZAZ2 were evaluated with UPDRS scores, significant and persistent improvements were found in the part II, parts II + III, and part IV scores (Table 2). Although some parts of UPDRS improved in week 1 in both the ZAZ2 and placebo groups, the improvement did not persist until week 13 (repeated-measure ANOVAs Table 2). There were significant differences in UPDRS Part II, Part II + Part III, and Part IV scores at week 13 between the ZAZ2 group and placebo group ($P < .05$, Bonferroni test; Table 2).

The local power-law exponent α, given by a slope of the log S versus log W $(S)^2$ relationship, characterizes the nature

Table 3: Effects on secondary symptoms of PD.

Group	Time	Nonfluent speech	Vertigo	Insomnia/ nightmare	Headache	Sweating or night sweats	Tiredness	Sense of cold	Dysuria
Placebo	0 weeks	1.12 ± 0.59	1.31 ± 0.97	2.67 ± 0.87	1.03 ± 0.75	2.13 ± 1.32	1.70 ± 0.97	1.78 ± 0.39	2.29 ± 1.02
	1 weeks	0.69 ± 0.32*	1.12 ± 0.69	2.40 ± 0.69*	0.96 ± 0.36*	1.87 ± 0.58	1.35 ± 0.69*	1.39 ± 0.81	1.69 ± 0.92*
	13 weeks	1.02 ± 0.36	1.28 ± 0.53	2.45 ± 0.38	0.99 ± 0.65	2.18 ± 0.56	1.58 ± 0.66	1.64 ± 0.58	2.18 ± 1.30
ZAZ2	0 weeks	1.08 ± 0.74	1.33 ± 0.83	2.77 ± 0.98	0.92 ± 0.56	2.11 ± 0.68	1.66 ± 0.57	1.90 ± 0.67	2.23 ± 0.69
	1 weeks	0.56 ± 0.28*	0.84 ± 0.26*#	2.03 ± 0.78*	0.64 ± 0.28*##	1.38 ± 0.69*#	1.21 ± 0.46*	1.48 ± 0.57*	1.43 ± 0.31*#
	13 weeks	0.65 ± 0.33*##	0.95 ± 0.37*#	1.73 ± 0.38*#	0.63 ± 0.19*#	1.48 ± 0.28*##	1.27 ± 0.51*#	1.58 ± 0.61	1.46 ± 0.36*##

Data presented are mean ± SD; $^{*}P < .05$; $^{**}P < .01$ compared with 0 weeks (repeated-measure ANOVAs). $^{\#}P < .05$; $^{\#\#}P < .01$ compared to placebo (Bonferroni test).

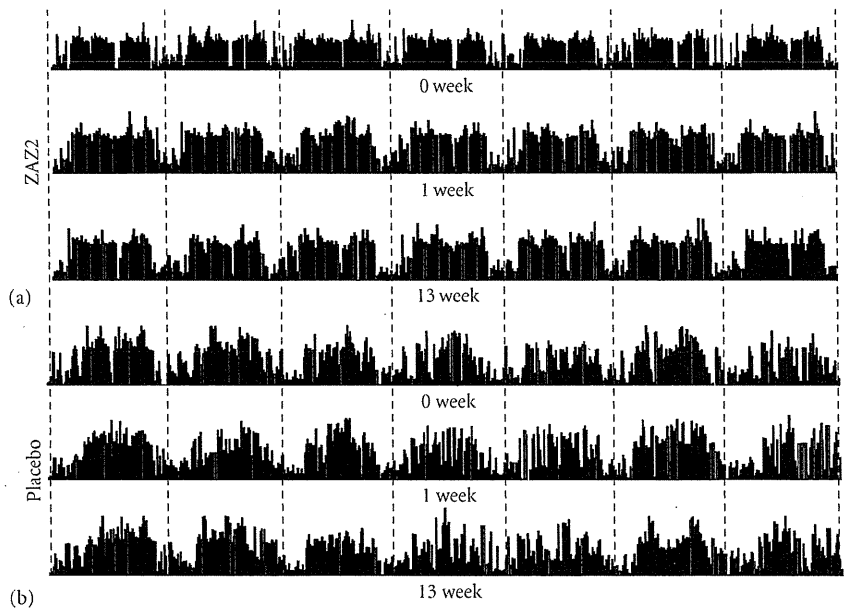

Figure 1: Daily profiles of AMI counts demonstrated the biological rhythm after granule ingestion in the ZAZ2 group (a) and in the placebo group (b). Each dash in the recordings represents midnight.

of "switching" patterns between high and low values in a statistical sense. The average wavelet coefficients exhibited linear relationships in the range of scales from 8 min to 35 min both for the ZAZ2 and placebo groups (Figure 2(b)). The local power-law exponent α values during both awake time and sleep time were significantly decreased both 1 week and 13 weeks after taking ZAZ2, but not after taking placebo granule (Table 2 and Figure 2(c), $P < .01$; Bonferroni test).

As the exploratory outcome of this study, most of the secondary symptoms were improved by the ZAZ2 treatment, whereas only a few symptoms in the placebo group were transiently improved in week 1 (Table 3).

4. Discussion

In this study, we demonstrate that ZAZ2, a TCM, ameliorates the disability of PD patients using the analysis of power-law temporal autocorrelation of the AMI records together with conventional UPDRS. Because a recent study indicated that improvement of the scores in UPDRS Part II reflect the long-term outcome of the patients [21], improvements of scores in UPDRS Part II, II + III, and IV likely reflect the beneficial effects of ZAZ2 on the patients' overall ADL as an endpoint of the treatment. ZAZ2 induced no significant adverse effects and was tolerable by more than 98% of the participants.

We previously demonstrated that the change in the local power-law exponent α is a quantitative predictor for evaluating the akinesia changes in PD [15, 20]. Because the α-values indicate persistency, lower α-values correspond to more frequent switching of behavior or higher physical activity [15, 20]. Therefore, the significant reduction in the α-values after ZAZ2 likely represents the improvement of motor function of the patients as a whole, which is in accordance with the improvement of scores of UPDRS Part II and Part II + Part III.

The effects of ZAZ2 are also demonstrated by the improvement in the scores for the secondary symptoms (Tables 2 and 3) and in the wake-sleep cycle (Figure 1) as

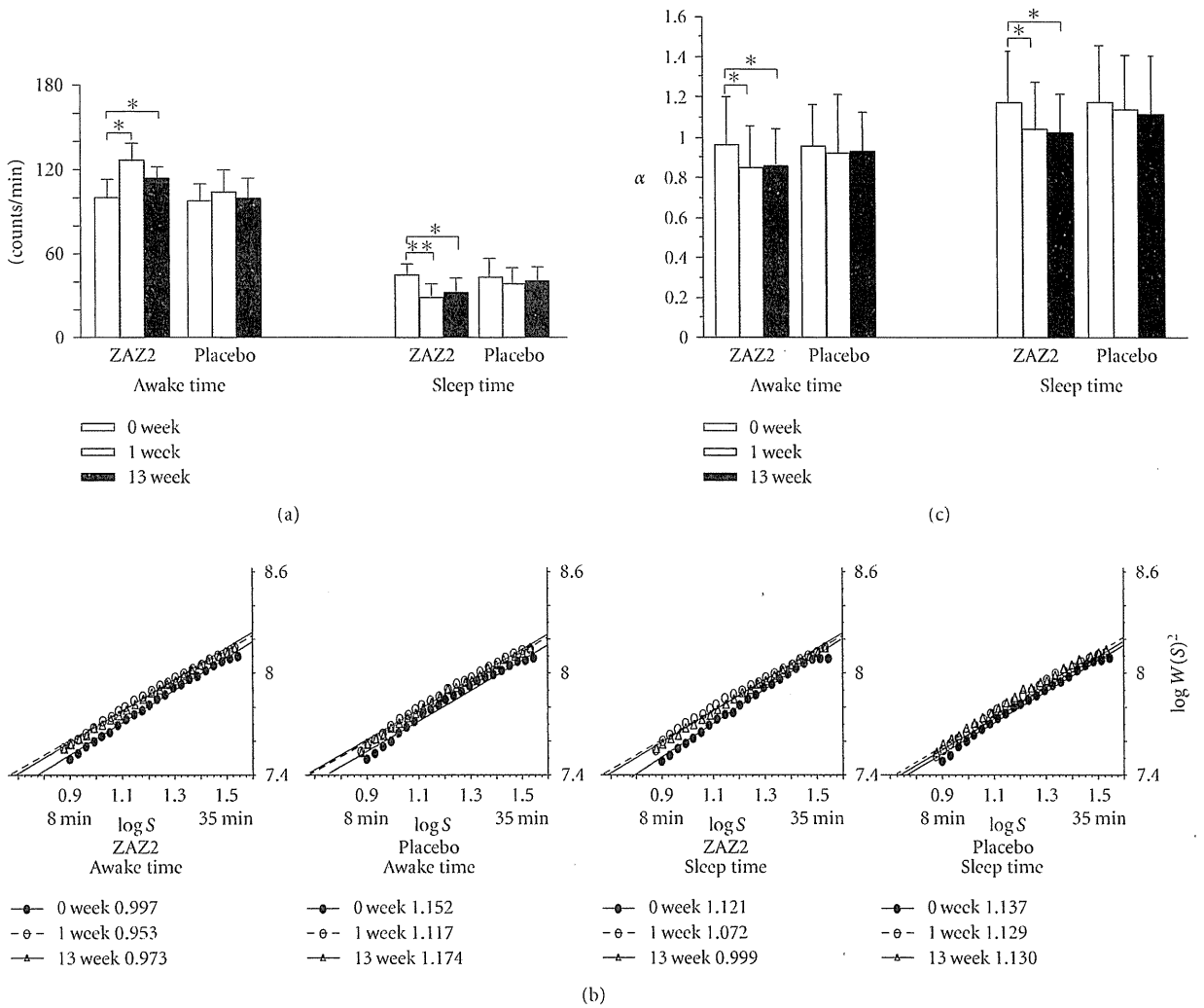

FIGURE 2: Before and after administration of granules of ZAZ2 and placebo for mean counts of physical activity (a), average wavelet coefficients, as a function of the wavelet scale for awake time and sleep-time, the slopes are power-law exponents, α (b), and comparisons of the mean α (c).

exploratory outcomes of this study. Indeed, activity during sleep time was markedly decreased and the local power-law exponent α was significantly decreased during sleep time as well as during awake time (Figures 2(a) and 2(c)). Because sleep disturbance, which is frequent among patients with PD, is thought to be due to disruption of the nighttime effects of levodopa [22], improvement of wake-sleep rhythm is likely a reflection of an improvement in parkinsonism. ZAZ2 may have beneficial effects on ADL by ameliorating the symptoms resulting from parkinsonism without exacerbating the L-dopa-induced adverse effects, as demonstrated in the improvement in scores of UPDRS Part IV.

TCM ameliorates various symptoms, particularly the ageing-related symptoms that are called *shen xu* (kidney deficiency) in Chinese [10, 11]. *Shen* (the kidney) denotes a functional visceral system (*zang*) that plays a central role in the regulation of growth, maturation, and ageing, and is subdivided into *shen yang* (kidney yang) and *shen yin* (kidney yin). Kidney yang can be described as the driving forces of all metabolic processes that improve the movements of the body. The production of kidney yin is considered to be effective at increasing nutrition to the muscles and improving the smoothness of the movements of the body by constituting the structive potential for the production of kidney yang. Based on this concept, TCM aims to potentiate a diminishing vitality of this transformative cycle caused by a decline of the essence (*jing*), which is stored in the kidneys and underpins the functions of both kidney yin and yang [23, 24].

Among the 14 components of ZAZ2, *Morindae officinalis radix and Desertliving Cistanche* might strengthen the "kidney yang" while *Asnaragus cochinchinensic, Cornus officinalis,* and *Rehmanniae radix* might increase the "kidney yin." The other components ameliorate secondary symptoms such as

headache, vertigo, and tinnitus (*Uncaria rhynchophylla and Rhizoma gastrodiae*), enhance the strength of the "kidney" (*Paeonia lactiflora, Puerariae radix, Salviae Miltiorrhizae radix, Curcuma longa Linn,* and *Rhizoma chuanxiong*), or increase blood circulation in the brain (*Arisaema consanguineum Schott* and *Acorus tatarinowii*). In addition, the inhibitory effects of TCM on fibril formation and inhibited Aβ aggregation (*Uncaria rhynchophylla*) [25], and on apoptosis and the neurobehavioral impairment of 1-methyl-4-phenylpyridinium ion (MPP^+)-induced PD model mice (*desertliving Cistanche*) [26, 27] and rats (*Uncaria rhynchophylla, Cornus officinalis, Rehmanniae radix,* and *Paeonia lactiflora*) [28] have been reported. Whether the herbs in ZAZ2 contain L-dopa or anticholinergic agents has not been demonstrated. Therefore, ZAZ2 may be potentially effective in the regulation of motor and various nonmotor symptoms of patients with PD without inducing the adverse effects of conventional anti-parkinsonism drugs. ZAZ2 is tolerable for long-term administration, and hence is likely a suitable choice as an additional drug for long-term control of the symptoms of PD.

Authors' Contribution

Weidong Pan participated in the entire study, formulated the study concept and design, provided statistical expertise, and assisted with drafting of the manuscript; Shin Kwak participated in the entire study and assisted with concept and design, and drafting of the manuscript; Yun Liu participated in part of content and data compilation; Yan Sun participated in part of content and data compilation; Zhenglong Fang participated in part of content and data compilation; Baofeng Qin participated in part of content and data compilation; Yoshiharu Yamamoto participated in part of content and critical reversion of the manuscript for important intellectual content.

Disclosure

All authors have no stock ownership in medically related fields, no consultancies, no advisory Boards, no partnerships, no grants, no intellectual property rights, no expert testimony, no employment, or no contracts as well as no royalties.

Conflict of Interests

The authors declared that there is no conflict of interest.

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A compound belonging to traditional Chinese medicine improves nocturnal activity in Parkinson's disease

To the Editor,

We describe a traditional Chinese medicine (TCM) that improves sleep disturbance in patients with Parkinson's disease (PD). Forty-eight PD patients (mean age, 65.3 ± 9.7 years, disease duration, 5.4 ± 6.9 years; mean ± SD) and 25 other patients as controls with neither sleep disturbance nor PD (66.8 ± 7.7 years) were enrolled. Patients took either Cerebralcare granules (CG) which are composed of 11 herbs [1] (CG, $n = 25$) or placebo granules ($n = 23$) in a randomized and double-blind manner for 6 weeks. The dosages of other anti-Parkinson medications were left unchanged from 4 weeks before and during the trial. All participants wore a motion logger (AMI) on their non-dominant wrist [2] for seven consecutive days. Sleep latency (SL), sleep efficiency (SE) [3], and the least active 5 h (L5) [4] of AMI records taken on one occasion in the control patients and on two occasions (before and 6 weeks after drug administration) in the PD patients were analyzed. We also determined the PD sleep scale (PDSS) score on two occasions in the PD patients. CG but not the placebo granules improved the daily profiles of activity counts as well as all these scores in PD patients to a level indistinguishable from those in controls (Fig. 1). The improvements in SL (18 min), median SE (16%), and the median L5 (−12.7) in the CG group were significantly larger than those in the placebo group (3.8 min, $p = 0.017$ and 1.9%, $p = 0.038$; 10.8, $p = 0.009$, respectively) (Fig. 1B). PDSS improved from 109.6 ± 20.8 to 122.8 ± 21.3 ($p = 0.043$, Fig. 1C). There were no significant changes from baseline in the UPDRS total in either group or in any motor scores in PD patients. The results indicate that this non-dopaminergic, non-gabaergic TCM medication may improve nighttime sleep problems in PD patients.

Disclosures

None.

Conflicts of Interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: doi:10.1016/j.sleep.2010.07.016.

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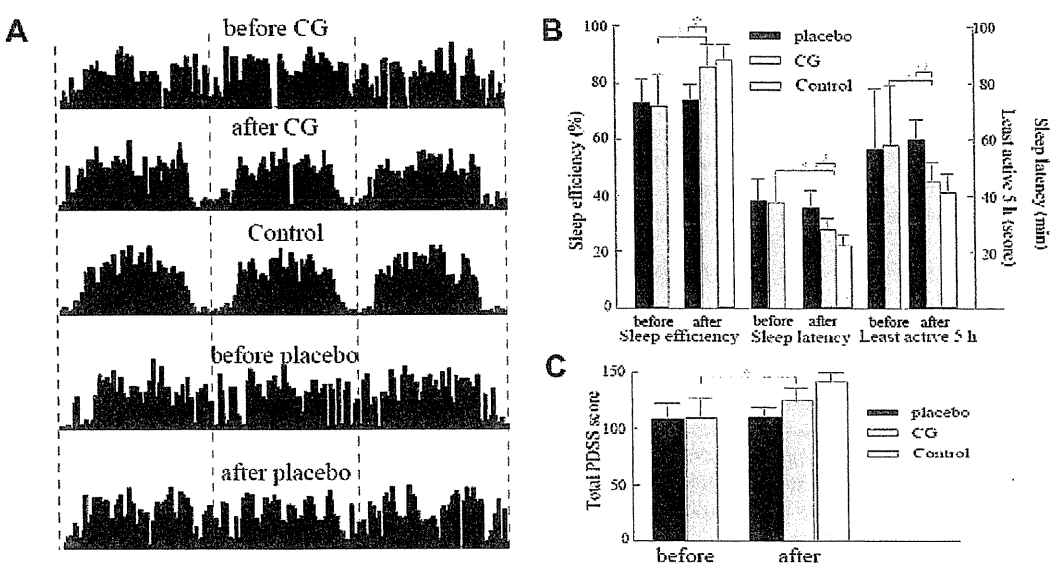

Fig. 1. (A) Daily profiles of AMI counts for three consecutive days before and after granule ingestion in the *cerebralcare granule* (*CG*) group, the placebo group, and the control group. Each dashed line in the recordings represents midnight. (B) Change from baseline in actigraphy measures at endpoint. Columns and bars (mean ± SD) indicate sleep efficiency (%), sleep latency (minutes), and the least active 5 h (counts/min). (C) PDSS scores in each group. *: $p < 0.05$; **: $p < 0.01$ (Mann–Whitney test).

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REM sleep related bradyarrhythmia syndrome: Vagal overactivity or dysfunction of the cardiac conduction system?

To the Editor:

Rapid eye movement (REM) sleep related bradyarrhythmia in young healthy adults seems to be associated with vagal over activation during REM sleep [1]. But the underlying mechanisms are unclear.

We performed polysomnography (PSG), analyzed spontaneous heart rate variability (HRV) using standard parameters in the frequency domain to detect sleep stage related changes in autonomic tone, and assessed the cardiac conduction system by transoesophageal electrophysiological cardiac testing (TECT) in a 47-year-old healthy female with reproducible REM sleep related type 1 second-degree atrio-ventricular (AV) block.

PSG revealed a sinus rhythm throughout the first part of the night. In the second part heart rhythm converted into continuous type 1 second-degree AV-block, starting at NREM–REM transition without any abnormal fluctuations in heart rate, respiratory events or body movements within the preceding 10 min. Apart from the heart block, PSG was without pathological findings.

HRV assessment showed a distinct increase in parasympathetic tone prior to the onset of the AV-block, contrary to sympathetic activation in NREM–REM transition and REM sleep without bradyarrhythmia.

TECT revealed a normal sinus node function but reduced global AV-conduction capacity (Wenckebach point without atropine: 690 ms, after 1 mg atropine: 620 ms). Exercise-ECG and echocardiography remained without pathological findings. Secondary origins for AV-conduction disturbances were excluded.

Normally, changes in autonomic tone vary from predominant parasympathetic tone during NREM sleep to sympathetic dominance during REM sleep [2]. Despite the sympathetic predominance, distinct vagal heart rate modulations are also present during NREM–REM transitions and REM sleep [3]. Associations between vagal activation and REM bradyarrhythmia has been demonstrated and were addressed to a heightened basal vagal tone [1]. Nevertheless, it remains unclear whether heightened basal vagal tone induces bradyarrhythmia exclusively in REM sleep and not in parasympathetic dominated NREM sleep. Clinical examination, Holter-ECG and TECT did not show increased basal vagal tone in our patient.

This raises the question if additional predispositional factors are necessary to induce REM bradyarrhythmias. Our patient showed a constitutional diminished AV-conduction capacity. The AV-node is modulated by the parasympathetic system, exerting an inhibitory influence on AV-node conduction [4]. Diminished AV-conduction capacity may predispose for the appearance of bradyarrhythmia during vagal activation [4,5].

We propose vagal activation alone is not sufficient to induce REM-bradyarrhythmias; rather a dysfunction of the cardiac conduction system is causally involved. Systematic verification of this assumption might become difficult due to the curiosity of the condition.

Potential conflicts of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: doi:10.1016/j.sleep.2010.11.006.

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Cerebrovascular Disease and Intracranial Artery Stenosis in Patients with Symptomatic Peripheral Artery Disease

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Background: Patients with symptomatic peripheral artery disease (PAD) have an elevated prevalence of internal carotid artery (ICA) stenosis and cerebral infarction, although the correlations between the severity of PAD and cerebral infarction, cerebral white matter lesion (WML), or intracranial or extracranial artery stenosis are unclear. *Methods:* We evaluated the prevalence of cardiovascular risk factors, cerebral infarction, and WML on magnetic resonance imaging and intracranial and extracranial carotid artery stenoses on magnetic resonance angiography in patients with symptomatic PAD (n = 136; males/females [M/F] 109/27) and a control group comprised of patients without PAD (n = 92; M/F 57/35). PAD was classified by Fontaine stage (stage II, n = 46; stage III, n = 20; stage IV, n = 70). Cerebral infarctions were classified into symptomatic or asymptomatic groups. WMLs were evaluated according to Fazekas stage. Artery stenosis was classified as normal (no stenosis), mild (stenosis <50%), moderate (stenosis ≥50%), severe (tight stenosis), and obstruction on magnetic resonance angiography. *Results:* Diabetes mellitus (DM), dyslipidemia, coronary artery disease (CAD), and chronic kidney disease (CKD), as well as symptomatic cerebral infarction and WML, were more frequent in patients with Fontaine III/IV PAD than without PAD. The prevalence rates of cerebral infarction and WML in patients with Fontaine stage II PAD were between those of the control and Fontaine III/IV PAD patients. Supraclinoid and cervical ICA stenoses (>50%) were more frequent in patients with Fontaine stage IV PAD than without PAD. *Conclusions:* Our results indicate that patients with advanced PAD have an increased prevalence of symptomatic cerebral infarction, WML, and intracranial and cervical ICA stenosis as well as DM, CAD, and CKD. **Key Words:** Cerebrovascular disease—intracranial artery stenosis—peripheral artery disease—magnetic resonance angiography—white matter hyperintensities.

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Peripheral artery disease (PAD) is a common manifestation of systemic atherothrombosis that is associated with significant morbidity and mortality. The major risk factors for PAD are the well-defined atherosclerotic risks, such as diabetes mellitus, cigarette smoking, advanced age, hyperlipidemia, and hypertension. PAD, cerebrovascular disease (CVD), and coronary artery disease (CAD) are manifestations of the same underlying condition (atherothrombosis). Therefore, they are closely associated. PAD was reported in 20% to 36% of patients with CVD.[1] Several long-term epidemiologic studies have shown that both asymptomatic and symptomatic PADs

are strongly associated with increased cardiovascular and cerebrovascular morbidity and mortality.[2]

Patients with symptomatic PAD have an elevated prevalence of carotid stenosis and ischemic stroke,[3] although the correlations between the severity of PAD and intracranial artery diseases, cerebral infarction, or cerebral white matter lesion (WML) are unclear. We examined the frequency of cerebral infarction, cerebral WML, and stenosis of intracranial artery as well as carotid artery stenosis in patients with symptomatic PAD.

Methods

Patients and Clinical Assessment

This was a retrospective study conducted at Asahikawa Medical University Hospital. One-hundred thirty-six consecutive patients with symptomatic PAD (males/females [M/F] 109/27) who were referred to the Division of Cardiovascular Surgery to assess the indication for surgical treatment for PAD were investigated in this study. In addition, 92 consecutive outpatients of the Division of Cardiovascular Medicine without PAD (M/F 57/35) who had at least 1 cardiovascular risk factor and/or cardiovascular disease and agreed to a magnetic resonance imaging (MRI) scan were recruited. All patients underwent MRI and magnetic resonance angiographic examinations to evaluate cerebrovascular diseases under informed consent. The cardiovascular risk factors and diseases included hypertension, diabetes mellitus, dyslipidemia, atrial fibrillation, CAD, chronic kidney disease (CKD), and cerebral infarction. Hypertension was defined as taking antihypertensive drugs, systolic blood pressure >140 mm Hg, or diastolic blood pressure >90 mm Hg. Diabetes mellitus was defined as taking antidiabetic agents or an HbA1c level ≥6.5%. Dyslipidemia was defined as taking lipid-lowering drugs, total cholesterol ≥220 mg/dL, low-density lipoprotein cholesterol ≥140 mg/dL, high-density lipoprotein cholesterol <40 mg/dL, or triglycerides ≥150 mg/dL. Atrial fibrillation was recorded as being present if there was a history or an electrocardiographic record of it. CAD was defined as having a history of angina or myocardial infarction (MI). CKD was defined as an estimated glomerular filtration rate of <60 mL per minute per 1.73 m^2. Cerebral infarction was classified into symptomatic and asymptomatic groups. Clinical symptoms and signs related to ischemic stroke were reported from neurologic examinations and/or clinical records. Symptomatic infarction was diagnosed when the symptoms correlated to the location of lesions on MRI. Cigarette smoking and alcohol consumption were excluded because data on smoking and alcohol consumption habits were available for <50% of patients. The clinical stage of symptomatic PAD was classified using the Fontaine staging system (stage II, n = 46; stage III, n = 20; stage IV, n = 70). Fontaine stages IIa and IIb included patients with intermittent mild and moderate to severe claudication, respectively; those with ischemic rest pain were classified as Fontaine stage III, and patients with ischemic ulceration and gangrene were classified into the Fontaine stage IV group. Patients with Fontaine stages IIa and IIb PAD were combined as Fontaine stage II for statistical analyses.

Magnetic Resonance Imaging and Magnetic Resonance Angiographic Examinations

MRI was performed using a 1.5-Tesla magnetic resonance unit (Signa EXCITE HD; GE Medical Systems, Waukesha, WI) using T1-weighted images (repetition time [TR]/echo time [TE] 500/15 msec), T2-weighted images (TR/TE 3300/97 msec), and fluid attenuated inversion recovery (FLAIR) images (TR/TE/inversion time 8000/120/2000 msec) in the axial orientation. The slice thickness was 5 mm, and the matrix size used was 320 × 256 pixels. MRA of the head was performed using a 3-dimension time-of-flight technique with a 19° flip angle, 6.3 ms TE, 26 ms TR, 1-mm section thickness covering 5 cm, and a 256 × 256 matrix encoding a 22-cm field of view. Source images were reconstructed to maximum intensity projection views of the intracranial vasculature. We evaluated cerebral infarctions and WMLs on MRI and carotid and intracranial artery stenosis on MRA.

We classified the PAD patients with cerebral infarction on MRI into 2 groups by lesion size (small 3-15 mm; large >15 mm). Cerebral infarction usually has an irregular edge that shows as high intensity on T2-weighted and FLAIR images and low intensity on T1-weighted images. Lesions <3 mm were excluded in this study.

WMLs were diagnosed as lesions with signal intensity higher than normal on both T2-weighted and FLAIR images as well as low intensity on T1-weighted images. WMLs in the periventricular and subcortical deep white matter regions were rated separately according to the Fazekas scale (total scores 0-6). The total Fazekas scores of WMLs in the periventricular and deep white matter regions were evaluated. Scores for periventricular hyperintensity (PVH) were: 0, absence; 1 "caps" or pencil-thin lining; 2, smooth "halo"; and 3, irregular PVH extending into the deep white matter. Scores for deep white matter hyperintensity (DWMH) were: 0, absence; 1, punctuate foci; 2, beginning confluence of foci; and 3, large confluent areas.[4]

We evaluated the supraclinoid internal carotid artery (ICA), M1 portion of the middle cerebral artery (MCA), and basilar artery in addition to the cervical ICA by MRA. Artery stenosis was classified into 5 grades according to the Cilostazol-Aspirin Therapy Against Recurrent Stroke with Intracranial Artery Stenosis (CATHARSIS) Study (ClinicalTrials.gov, identifier NCT00333164) as: normal, no evidence of stenosis (grade 0); mild stenosis, <50% stenosis (grade 1); moderate stenosis, >50% stenosis (grade 2); severe stenosis, partial signal loss with the

distal flow signal (grade 3); and occlusion, no distal flow signal (grade 4) in at least 2 dimensions on MRA (Figs 1 and 2).[5]

Two observers (a neurologist and a radiologist other than the present investigators) read the MRI and MRA images in a blinded manner. The interobserver agreement was calculated.

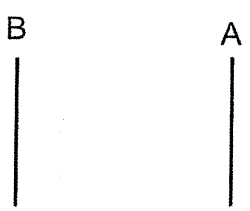
Statistical Analysis

The Mann–Whitney *U* and post-hoc analysis (Bonferroni) tests were used to compare age, sex, cardiovascular risk factors, cerebral infarction, WML, and intracranial and cervical carotid artery stenosis between groups. Multiple logistic regression analysis was performed to examine the possible factors associated with PAD. All analyses were performed with SPSS II (SPSS, Inc, Chicago, IL).

Results

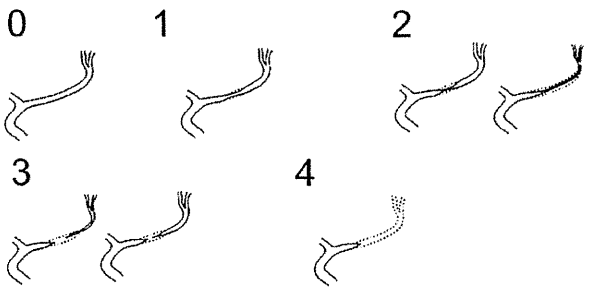
Table 1 summarizes the background clinical characteristics of the patient controls and symptomatic PAD patients. There was no significant difference in the frequency of hypertension between the patient controls and symptomatic PAD patients, although it was more frequent in the Fontaine III patients than in the Fontaine II patients, possibly because the Fontaine III patients were slightly older. Diabetes mellitus was observed more frequently in PAD patients (65.0%) than in the patient controls (17.0%). On the other hand, dyslipidemia was observed less frequently in symptomatic PAD patients (22.8%) than in the patient controls (44.7%). CAD was more frequent in symptomatic PAD patients (49.3%) than in the controls (14.9%). CKD was also more frequently observed in the symptomatic PAD (52.2%) patients than in the controls (8.5%).

The interobserver agreement on MRI/MRA readings was >90%. The final ratings were made by consensus of the 2 observers reviewing the images. Total (symptomatic or asymptomatic) cerebral infarctions were more frequent in the symptomatic PAD patients (64.0%) than in the controls (29.0%; $P < .001$). When cerebral infarction was



Stenosis rate (%) = (b-a)/bx100
A: Vessel diameter of most stenotic portion
B: Normal vessel diameter

Figure 1. *Formula to measure stenosis rate on magnetic resonance angiography.*



0 Normal: no evidence of stenosis
1 Mild stenosis: less than 50% stenosis
2 Moderate stenosis: more than 50% stenosis
3 Severe stenosis: partial signal loss with the distal flow signal
4 Occlusion: no distal flow signal

Figure 2. *Grading of intracranial artery stenosis.*

classified by size, both symptomatic small (25.7%) and symptomatic large (25.7%) infarctions were more frequently observed in the PAD patients than in the controls (4.3%; $P < .001$ and 7.6%; $P < .001$, respectively). There were no significant difference in the frequency of asymptomatic large or small infarctions between the PAD patients and the controls.

The Fazekas scores of the Fontaine stage III (2.8 ± 1.3; mean ± SD) and IV (2.8 ± 1.4) groups were higher than that of the control (1.8 ± 1.2) group. The Fazekas score of the Fontaine stage II (2.2 ± 1.0) group was between that of the control group and the Fontaine stage III and IV groups. The PVH score of the Fontaine stage IV (1.5 ± 0.7) group was higher than that of control (1.1 ± 0.7) group. There was no significant difference in PVH score between control and Fontaine stage II/III groups. The DWMH score of all the Fontaine stage groups was higher than that of the control group. There was no significant difference in the DWML scores among all the Fontaine stage groups.

MRA revealed that the frequency of supraclinoid ICA stenosis (>50%) was higher in the Fontaine stage IV PAD patients (23.9%) than in the controls (3.2%). There was no significant difference in the frequency of stenosis of the M1 portion of the MCA between the PAD patients and the controls. A significant basilar artery stenosis was present only in 6% of the Fontaine stage IV patients. A cervical ICA stenosis was observed more frequently in the combined PAD patients (21.8%) than in the controls (2.5%). When PAD patients were divided by Fontaine stage, cervical ICA stenosis was more frequently observed in the Fontaine stage II (23.8%) and IV (26.2%) patients than in the controls. No significant stenosis (>50%) of the cervical ICA was present in the Fontaine stage III PAD patients.

Differences in the frequencies of cardiovascular diseases and risk factors were analyzed using multiple logistic regression analysis (Table 2). Significant odds ratios (95% CI) for the PAD versus the control were: age (>65