CHZ does not have any notable adverse effects

To be used therapeutically, systemic administration of CHZ should be devoid of side effects. We performed a number of experiments to examine whether CHZ adversely affected wild-type C57BL mice. 17 adult male mice (age-matched with *tottering* mutant mice) were divided into two groups. One group was given normal tap water, whereas the second group was supplied with tap water containing 30 mM CHZ. Note that the concentration of CHZ used for these experiments was twice as high as that used in the *tottering* mice to improve their motor performance. After one week of treatment with CHZ, a battery of tests was performed to examine the consequence of administration of CHZ on muscle strength, gross motor performance, and a simple cognitive task. The mice continued to receive CHZ during the tests.

CHZ does not affect muscle strength

CHZ is often prescribed as a muscle relaxant (Chou et al., 2004). Even though it is currently thought that CHZ produces its effects by acting centrally (Chou et al., 2004), we examined whether at the concentrations used it caused muscle weakness. We employed a simple *hanging test*. Mice were placed on a wire mesh which was then inverted so that the mice were hanging upside down. The length of time before the mouse let go and fell was measured (Crawley, 1999). As can be noted in Figure 1A CHZ did not reduced the time that the

animals stayed on the mesh suggesting that CHZ had no adverse effects on muscle strength.

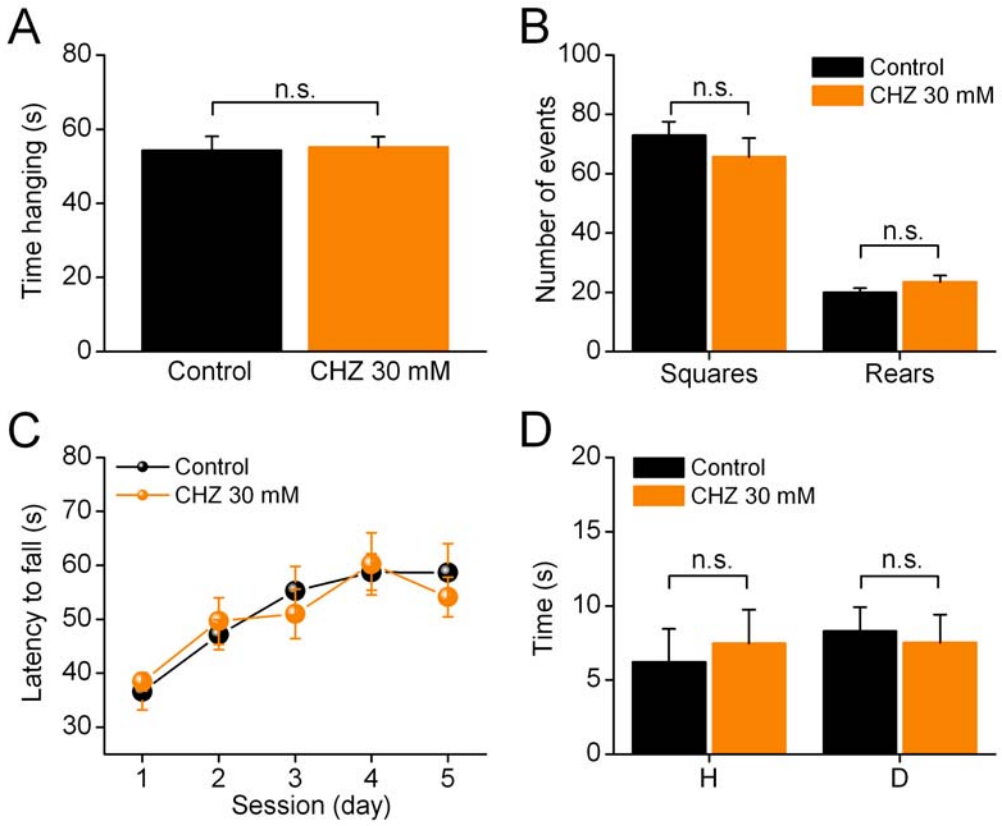

Figure 1: CHZ has no adverse effects
Average data obtained in the behavioral tests conducted on mice treated with CHZ or normal tap water. (A) Hanging test, (B) Open field test, (C) Accelerating rotarod, (D) Object recognition test, H represents the habituation index and D the discrimination index.

General tests for motor performance

To evaluate gross motor function we performed two tests: *open field* and an *accelerating rotarod* paradigm. The *open field test* was used to examine overall locomotor activity (Crawley, 2008). Using a rectangular box divided into 8 identical squares we counted the number of squares that a mouse entered in a 3 min period. The number of times each mouse reared during each session was

also quantified. As depicted in Figure 1B, CHZ had not effect on either of these two parameters.

The mice were also examined on the rotarod using the same protocol used with the mutant *tottering* mice. As can be seen in Figure 1C, CHZ did not change the maximum proficiency achieved or the slope of the learning curve demonstrating that it did not affect motor coordination or learning.

Object recognition

The *object recognition test* is often used to evaluate cognitive function (Crawley, 2008; Ennaceur and Delacour, 1988). This test consisted of two sessions: in the first session each mouse was exposed to two identical objects, in the second session one of the objects was replaced with a novel one. Two indices were calculated: the index of habituation (H), and the index of discrimination (D). The index of habituation reflects how well the mice remember the first object, and the index of discrimination represents their ability to discriminate a novel object. Figure 1D shows that treatment with CHZ did not affect either of these two indices.

Methods

Hanging test. Each mouse was suspended in an elevated metal mesh for a maximum time of 60 seconds per trial (Crawley, 1999). The latency to fall was measured and averaged in 3 trials.

Open field test. Mice were placed in the center of a 32 ×18 cm rectangular box divided into 8 identical squares. The animals were allowed to move freely for 3 minutes and the total number of squares that each mouse entered was quantified. The number of rears (both front paws off the ground, against the wall or standing) was also counted.

Accelerating Rotarod. The apparatus consisted of a 3 cm diameter rotating rod (Rotamex-5, Columbus Instruments) elevated 55 cm above a covered platform. Each trial started with the rod in stationary position accelerating at a rate of 0.1 cm/s. Speed and latency to fall of the animals were automatically recorded by a computer. Mice were tested in 5 consecutive days with 10 daily trials.

Object recognition test. The mice were placed in the same arena used for the open field test. The object recognition test consisted of two sessions(Ennaceur and Delacour, 1988), in the first session each mouse was exposed to a pair of identical objects, and in the second session one object was replaced with a novel one. The inter-session interval was 1 hour. The time that the animals explored each object was measured (exploration was defined as directing the nose to the object or touching it with the nose(Ennaceur and Delacour, 1988)). Two parameters were calculated: habituation index (H) and discrimination index (D). H was calculated by subtracting the total exploration time in session 2 from the total exploration time in session 1. D was the difference between exploration time of new object with that of the old one in session 2.

Chlorzoxazone (CHZ) was orally administrated by adding it to the drinking water. The solution was prepared fresh daily as described in Methods of the

main manuscript. Because rodents are nocturnal, all behavioral tests were carried out during their dark cycle. All data are reported as Mean ± S.E.M. Data were analyzed using one-way ANOVA and considered not to be statistically significant if $p>0.05$.

References

Chou R, Peterson K, Helfand M (2004) Comparative efficacy and safety of skeletal muscle relaxants for spasticity and musculoskeletal conditions: a systematic review. J Pain Symptom Manage 28: 140-175.

Crawley JN (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 835: 18-26.

Crawley JN (2008) Behavioral phenotyping strategies for mutant mice. Neuron 57: 809-818.

Ennaceur A, Delacour J (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31: 47-59.