Management of sleep/wake cycles improves cognitive function in a transgenic mouse model of Huntington’s disease

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ABSTRACT

Normally, mice sleep during the day and are active at night. In Huntington’s disease mice (R6/2 line) this circadian pattern disintegrates progressively over the course of their illness. Cognitive decline and apathy in R6/2 mice can be improved with sleeping drugs, suggesting that sleep disruption contributes to their neurological decline. We wondered if wakefulness was equally important. Here, we used two drugs to manage sleep/wake cycles in R6/2 mice, Alprazolam (to put them to sleep) and Modafinil (to wake them up). We found that both drugs improved cognitive function and apathy, but had a stronger effect when used in combination. Remarkably, beneficial effects on cognitive performance were also seen in vehicle-treated cage-mates of Alprazolam/Modafinil-treated mice, suggesting that behavioral intervention to regularize sleep/wake activity might be therapeutically useful. We suggest that focused management of sleep and wakefulness will slow the progression of cognitive decline and apathy in neurological conditions where sleep is disordered.

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1. Introduction

Although best known as a movement disorder, HD involves a panoply of other symptoms that includes cognitive decline, psychiatric disorder (for references, see Bates et al., 2002), and sleep and circadian abnormalities (Arnulf et al., 2008; Morton et al., 2005; Pallier et al., 2007; Petersén et al., 2005). Regular sleep/wake cycles are central to normal neurological function (Malik and Kaplan, 2005; Maquet, 1995; Roth and Ancoli-Israel, 1999; Zammit et al., 1999; Zisapel, 2007), and their disruption is deleterious to cognitive function (Cirelli, 2005; Durmer and Dinges, 2005; Harrison and Horne, 2000; Zammit et al., 1999). In HD patients, disrupted sleep may not only contribute to disease progression, it may also have a knock-on effect on the quality of sleep of the partner or carer (Gruffydd and Randle, 2006; Morton et al., 2005). The inability of carers to cope with nocturnal sleep dysfunction is one of the most common reasons for institutionalization of patients with neurodegenerative disease (Bianchetti et al., 1995; Hatfield et al., 2004). Therefore, improving sleep/wake activity patterns in HD patients might not only improve their cognitive function, it may also improve the daily living and quality of life of the patients and their carers.

We have shown that the sleep disruption in HD patients is mirrored in a transgenic mouse model of HD, the R6/2 mouse (Morton et al., 2005). R6/2 mice show a progressive neurological phenotype in which overt motor signs are observed from around 8 to 10 weeks of age (Carter et al., 1999; Mangiarini et al., 1996). Interestingly, age of onset, progression of the HD behavioral phenotype, and lifespan depend on the CAG repeat length, as shown recently in our laboratory, where increasing the repeat length of R6/2 mice delayed onset of disease and prolonged survival (Morton et al., 2009). In mice with CAG repeats of 150–200, cognitive deficits are measured as early as 4 weeks of age, and decay of learning can be tracked in R6/2
mice using cognitive tasks sensitive to fronto-striatal and hippocampal function (Ciamei and Morton, 2008; Lione et al., 1999; Murphy et al., 2000). R6/2 mice also show progressively disrupted day-night activity patterns, with increased daytime activity and a concurrent decrease in nocturnal activity that eventually leads to a complete disintegration of circadian behavior; these are accompanied by a dysregulation of circadian clock genes (Morton et al., 2005; Pallier et al., 2007). Daily treatment with a hypnotic dose of a short-acting benzodiazepine, Alprazolam, improved the cognitive performance of R6/2 mice in a two-choice visual discrimination task, the two-choice swim tank task (Pallier et al., 2007). Acquisition of the discrimination in this task requires implicit learning of a stimulus-response reinforcement association. Implicit learning requires striatum integrity (Packard and Knowlton, 2002), and HD patient are impaired in a number of tasks of non-declarative memory (Heindel et al., 1989; Knopman and Nissen, 1991; Lawrence et al., 2000). Therefore, the two-choice swim tank task is ideal to test striatal dysfunction in R6/2 mice. In our previous study, we attributed the learning improvements seen in R6/2 mice on this task to drug-imposed sleep. We wondered whether improving wakefulness during the active period would be as beneficial to cognitive function of R6/2 mice as sleep was when it was imposed during the sleep period. To improve wakefulness in R6/2 mice, we used Modafinil, a wake-promoting drug that is prescribed to treat excessive sleepiness associated with narcolepsy, idiopathic hypersomnia, and shift work sleep disorder (for references, see Ballon and Feifel, 2006).

2. Results

2.1. Alprazolam and Modafinil improved reversal learning deficits in R6/2 mice

As expected, wild-type (WT) mice learned both the visual discrimination of the two-choice swim tank and its

![Fig. 1](image-url) - A combination of Alprazolam and Modafinil improves performance in the two-choice swim tank task. Percentage of correct choices made during the first 10 trials in the two-choice swim tank, during acquisition and reversal, by drug-treated (filled symbols) compared to vehicle-treated (open symbols) mice of the Alprazolam (A; squares, WT mice; triangles, R6/2 mice), Modafinil (B; triangles, R6/2 mice) and Alprazolam/Modafinil (C; diamonds, R6/2 mice) treatment groups. Mouse age (wk) is shown on the X axis. Symbols indicate means ± SEM of each genotype for each treatment group. Where error bars are not visible, they are obscured by the size of the symbols. Asterisks indicate significant differences between drug-treated mice and vehicle-treated controls (*p < 0.05; **p < 0.01). Alprazolam (n = 16 WT and n = 16 R6/2, acquisition; n = 16 WT and n = 13 R6/2, reversal), vehicle for Alprazolam (n = 16 WT and n = 16 R6/2, acquisition; n = 16 WT and n = 14 R6/2, v = reversal), Modafinil (n = 14 R6/2, acquisition; n = 12 R6/2, reversal), vehicle for Modafinil (n = 15 R6/2, acquisition; n = 14 R6/2, reversal), Alprazolam and Modafinil (n = 15 R6/2, acquisition and reversal) or Alprazolam vehicle and Modafinil vehicle (n = 14 R6/2, acquisition and reversal).
reversal, reaching criterion by day 5 during acquisition and by day 18 during reversal (open squares, Fig. 1A). Vehicle-treated R6/2 mice all learned the discrimination, as demonstrated by the significant drop in performance between the last day of acquisition and the first day of reversal learning (Alprazolam group; t = 5.15, p < 0.001; Modafinil group; t = 4.98, p < 0.001; Alprazolam/Modafinil group; t = 11.96, p < 0.001) (Fig. 1). However, performance during acquisition differed between the 3 groups of vehicle-treated R6/2 mice. Vehicle-treated R6/2 mice from the Alprazolam group (open triangles, Fig. 1A) did not reach criterion (arbitrarily set at 80%), while vehicle-treated R6/2 mice from the Modafinil group (open triangles, Fig. 1B) and from the Alprazolam/Modafinil group (open diamonds, Fig. 1C) reached criterion by days 7 and 6 respectively. By contrast, all 3 groups of vehicle-treated R6/2 mice had pronounced impairments in reversal learning. None of the groups had reached criterion by day 25 (Fig. 1 and Supplementary Fig. 1A), although the mean performance during reversal depended on the treatment group (see below).

Chronic treatment with Alprazolam had neither a beneficial nor a deleterious effect on the performance of the WT mice during acquisition of the two-choice visual discrimination (filled squares, Fig. 1). During reversal learning, the drug-treated WT mice performed significantly better than their vehicle-treated control mice on the second day (trial×treatment; F[12,324]=2.59, p < 0.01), but overall the performances of the 2 groups were similar (treatment; F[1,27]=0.47, p=0.50). There was no overall effect of Alprazolam on the performance of R6/2 mice during acquisition of the two-choice swim tank task, although, consistent with the results of our previous report (Pallier et al., 2007), Alprazolam-treated R6/2 mice tended to perform better than vehicle-treated R6/2 mice over the last 7 days of acquisition (treatment; F[1,30]=2.99; p=0.09) (Fig. 1A). Performance of drug-treated R6/2 mice from the Modafinil and Alprazolam/Modafinil groups was the same as that of their vehicle-treated control mice during acquisition. Over the first 13 days of reversal learning, neither the Alprazolam-treated (filled triangles, Fig. 1A) nor the Modafinil-treated (filled triangles, Fig. 1B) R6/2 mice performed better than their respective vehicle-treated control groups, and none of the groups reached criterion. We continued training these mice for a further 10 days. R6/2 mice treated with Alprazolam alone had still not reached criterion by day 35, although they performed significantly better than the vehicle-treated R6/2 mice over this period (treatment; F[1,25]=6.14, p < 0.05). By contrast, R6/2 mice treated with Modafinil alone reached criterion by day 34 (treatment; F[1,24]=4.62, p < 0.05). However, when Alprazolam and Modafinil were given together (filled diamonds, Fig. 1C), there was a much greater improvement of performance of the R6/2 mice than when either drug was given separately, with Alprazolam/Modafinil-treated R6/2 mice reaching criterion by day 23 (treatment; F[1,27]=7.56, p = 0.01) (Fig. 1C; see also Supplementary Fig. 1B). Although the Alprazolam/Modafinil-treated R6/2 mice were slower to reach criterion than the WT mice, the fact that they learned to perform the reversal phase of the task is remarkable given the relatively small effects of each individual drug when given separately. Because these mice were still in good physical condition at the end of the experiment, we decided to test them in a task that is classically used to assess locomotor reactivity to novelty and anxiety levels (the open-field test) and on accelerating rotarod (8–40 rpm within 10 min), at 18 weeks of age. Results of the open-field test showed that performance of drug-treated R6/2 mice was not significantly different to that of vehicle-treated mice, both when measures specific to locomotor activity and those classically used as indexes of anxiety were considered (data not shown). We had previously obtained similar results with R6/2 mice treated chronically with Alprazolam alone (Pallier et al., 2007), and the results were confirmed in the elevated zero maze, a classic test of anxiety (data not shown). On accelerating rotarod, Alprazolam/Modafinil-treated R6/2 mice showed no sign of significant motor improvement (data not shown). However, there was a trend towards improvement over the first 2 trials, suggestive of improved procedural (implicit) learning, a result
that supports the results obtained in the two-choice swim tank.

2.2. Reversal learning impairment of the vehicle-treated R6/2 mice depended on the treatment group

During reversal learning, the mean performance of the vehicle-treated R6/2 mice of the Alprazolam group and of the Modafinil group remained at chance after 13 days of training, by day 25 (Figs. 1A, B). By day 35, only 2/14 vehicle-treated R6/2 mice of the Alprazolam group had reached criterion. This is consistent with our previous findings, namely that R6/2 mice have difficulties reversing a two-choice visual discrimination, even with prolonged training (Morton et al., 2006; Pallier et al., 2007). However, although the group as a whole had not reached criterion by day 35, 10/14 vehicle-treated R6/2 mice of the Modafinil group had done so. By contrast, the performance of the vehicle-treated R6/2 mice controlling for the Alprazolam/Modafinil group was significantly better than that of either of the vehicle-treated groups controlling for the single treatments. Group performance of the vehicle-treated R6/2 mice from the Alprazolam/Modafinil group was significantly above chance from day 20 of reversal training onwards, and the average performance of the group approached criterion by day 25 (76±4%) (Fig. 1C; see also Supplementary Fig. 1A). On this day, 8/14 individual vehicle-treated R6/2 mice had reached criterion (note that these mice were not tested for 35 days, since the Alprazolam-treated mice were controls had already reached criterion by day 23).

2.3. Alprazolam/Modafinil treatment improved the rousability of R6/2 mice

In addition to having a beneficial effect on cognition, the Alprazolam/Modafinil treatment had a significant beneficial effect on the rousability of R6/2 mice (Fig. 2). With increasing age, vehicle-treated R6/2 mice showed reduced reaction to cage disturbance (Figs. 2A, C). However, there was a long-term beneficial effect on the pre-dosing rousability of the R6/2 mice after treatment with both Alprazolam and Alprazolam/Modafinil. Compared with vehicle-treated R6/2 mice, more Alprazolam-treated and Alprazolam/Modafinil-treated R6/2 mice were out of their nest 30 s, 60 s and 5 min after cage opening (observation time×treatment; F[3,24]=27.85, p<0.001; Alprazolam group; observation time×treatment; F[3,24]=15.82, p<0.001; Alprazolam/Modafinil group) (Fig. 2A, C). As was seen in our previous report (Pallier et al., 2007), R6/2 mice treated with Alprazolam alone showed residual drowsiness 4 h after dosing, especially during the first 2 weeks of treatment and at 15 weeks of age (Fig. 3A). By contrast, 4 h after injection of the hypnotic dose of Alprazolam, R6/2 mice treated with Alprazolam/Modafinil did not show any residual drowsiness; on the contrary, their rousability was increased compared to that of the vehicle-treated control mice (observation time×treatment; F[3,24]=5.30, p<0.01) (Fig. 3B). This effect was seen throughout the course of the study.

The effect of Modafinil on rousability was very pronounced (Fig. 2B). The rousability of both the Modafinil-treated and vehicle-treated R6/2 mice controlling for this group was indistinguishable from that of the WT mice (Fig. 2A), with no mice remaining in their nest after 60 s of cage opening.

2.4. Dosing regime influenced body weight gain

Vehicle-treated R6/2 mice of the Alprazolam- and Modafinil-treatment groups gained weight over the first 4 weeks of treatment (mean body weight peaked at 12 weeks of age), and then gradually lost body weight until the end of the experiment. By contrast, vehicle-treated R6/2 mice of the Alprazolam/Modafinil-treatment group significantly lost body weight over the first 4 weeks of treatment (week of treatment×treatment group; F[16,312]=2.11, p<0.01; data not shown). At 12 weeks of age (first week of testing in the two-choice swim tank), these mice showed a mean 4±1% weight loss, as opposed to 2±1% and 1±1% weight gain in the vehicle-treated R6/2 mice of the Alprazolam- and Modafinil-treatment groups, respectively. After this initial suppression of body weight gain, that was maximal at 12-weeks of age, the weight of the vehicle-treated R6/2 mice of the Alprazolam/Modafinil-treatment group increased again, slightly but steadily, to peak at 16 weeks of age. At 17 weeks (last week of reversal training), these mice had lost a mean 4±3% of body weight, as opposed to a 18±3% and 6±2% body weight loss in the vehicle-treated R6/2 mice of the Alprazolam- and Modafinil-treatment groups, respectively.
The body weights of Alprazolam-treated and Modafinil-treated R6/2 mice were not significantly different to these of their respective vehicle-treated controls. However, both Alprazolam-treated WT mice and Alprazolam/Modafinil-treated R6/2 mice were significantly lighter than their respective vehicle-treated controls. In these drug-treated groups, body weight loss, relative to vehicle-treated controls, started after 3–4 weeks of treatment (week of treatment x treatment; F[8,232]=4.56, p < 0.001; WT mice, Alprazolam group; treatment; F[1,24]=9.09, p < 0.01; Alprazolam/Modafinil group; data not shown).

3. Discussion

We show that promoting wakefulness with the drug Modafinil has beneficial effects on the cognitive function and rousability scores of R6/2 mice that are additional to those mediated by the sleep imposition with Alprazolam.

It seems unlikely that the cognitive improvements were mediated solely by a direct effect on cognition of the drugs themselves, since the major action of the drugs we used was to modify sleep or wakefulness and the treatments had a knock-on effect on the cognitive performance of the vehicle-treated mice. Rather, it appears that pharmacological imposition of sleep and/or wakefulness mediated the improvements in cognitive function. A beneficial effect of regular sleep/wake rhythms on cognition is consistent with data taken from human studies. Research conducted with healthy individuals deprived of sleep has shown that combining sleep with Modafinil had the best effect on cognitive performance (Batéjat et al., 2006; Batéjat and Lagarde, 1999).

Two complementary possibilities might explain why the treatment combining Alprazolam and Modafinil was more beneficial to R6/2 mice than either treatment alone. First, each drug could have had independent effects on the re-entrainment of the sleep/wake cycle. There is evidence that benzodiazepines administered at hypnotic doses can entrain circadian rhythms in Syrian hamsters (Biello and Mrososky, 1993), squirrel monkeys (Mistlberger et al., 1991), and humans (Buxton et al., 2000). In humans and in rodents, the cognitive-enhancing properties of Modafinil are well documented (for review, see Minzenberg and Carter, 2008). However, a direct pharmacological effect of Modafinil through increased alertness/vigilance seems unlikely, given that the elimination half-life of Modafinil is 1–3 h in mice (Moachon et al., 1996) and that rousability was tested 14–15 h after drug administration and cognitive performance was tested 19–24 h after drug administration. Further, in a pilot study, we examined the acute effect of Modafinil by testing R6/2 mice in the two-choice swim tank 30 min after administration of 32, 64, or 100 mg/kg Modafinil. None of the drug-treated R6/2 mice showed any significant improvement in this task (data not shown). As a wake-promoting drug, Modafinil also targets hypothalamic structures involved in the regulation of sleep, wakefulness and circadian rhythms (Chapotot et al., 2003; Lin et al., 2000). Although chronobiotic properties of Modafinil have not yet been tested in mice, it is possible that Modafinil increases the sensitivity of the circadian pacemaker to nonphotic “zeitgebers”, as suggested by Webb et al. (2006). Second, while Alprazolam and Modafinil could act separately to reinstate the circadian drive for sleep and wakefulness in R6/2 mice, they may also have downstream effects that interact with each other. The wake and sleep phases of the circadian cycle are tightly linked (Fuller et al., 2006). For example, the daily imposition of wakefulness in the R6/2 mice could have a knock-on effect on the quality of their sleep, effectively enhancing the restorative effect of drug-induced sleep, and vice versa. The fact that chronic treatment with Alprazolam improved the pre-dosing rousability of R6/2 mice suggests that chronic Alprazolam increased the likelihood of the mice being more behaviorally “engaged” during their active phase. In humans, Modafinil does not cause sleep rebound as compensation for its waking properties in the absence of sleep debt (Batéjat et al., 2006). However, in mice, Modafinil can induce sleep rebound (Kopp et al., 2002), albeit at a dose that is >3 times higher than the one we have used here. Therefore, the possibility exists that Modafinil exerts a rebound effect on sleep in sleep-deprived R6/2 mice. This may be beneficial to animals whose sleep is disrupted pathologically.

It is possible that the improvements seen in the cognitive performance of the vehicle-treated cage-mates were due to re-entrainment of the biological clock of the R6/2 mice by the vehicle administrations themselves. However, this is unlikely, since the performance of the vehicle-treated R6/2 mice for the alprazolam group was worse than those of the other two vehicle treatment groups. Alternatively, combination administration of the vehicles (2.5% methanol and saline in the morning and 4% corn flour solution in the evening) may have had a beneficial effect on the general health of R6/2 mice (e.g., through re-hydration — see Wood et al., 2008). This might have explained the better performance in the two-choice swim tank of the vehicle-treated R6/2 mice from the Alprazolam/Modafinil group. However, the weight data we collected did not support this idea. Vehicle-treated R6/2 mice of the Alprazolam/Modafinil-treatment group showed significant loss of body weight over the first 4 weeks of treatment, whereas their cognitive performance eventually improved. Differences in the handling regime might have explained the differences in weight between groups, since dosing rodents twice daily induces a state of mild chronic stress (Flemmer and Dilsaver, 1989) with moderate suppression of body weight gain (Izumi et al., 1997). However, suppression of body weight gain was more pronounced with the drug treatment that was best at improving cognitive function in R6/2 mice, and was also seen in the Alprazolam-treated WT mice. Recently, both in mice and man, it was shown that chronic stress induces a shift towards stimulus-response learning when both a spatial and a stimulus-response strategy are available to solve a learning task (Schwabe et al., 2008). A state of mild chronic stress could explain better performance in the two-choice swim tank of the vehicle-treated R6/2 mice of the Alprazolam/Modafinil-treatment group. However, this is unlikely to account for the improved performance in the swim tank throughout acquisition and reversal, since the weight of these mice increased again after the initial suppression of body weight gain. The most likely explanation is that there was a “knock-on” effect of the behavior of the drug-treated R6/2 mice on the behavior of the vehicle-treated cage-mates. There is good evidence that social stimuli can promote synchronous
activity among members of a colony, and act as zeitgebers to substitute for, or to reinforce the actions of light on the circadian phase (for review, see Mistlberger and Skene, 2004). Therefore, sleeping behavior of the Alprazolam-treated R6/2 mice could act as a cue to promote huddling and better sleep for all mice in the cage. Similarly, the wake-promoting effect of Modafinil treatment on the drug-treated R6/2 mice would reduce the chance of rest and increase the opportunity for social interactions of the vehicle-treated R6/2 mice during their active phase. It is known that social interactions alter the expression of daily rhythms by directly elicitng behaviors (for references, see Mistlberger and Skene, 2004) although, to our knowledge, entrainment of circadian activity by the behavior of drug-treated cage-mates has not been examined previously, either in normal mice or in mice with neurological abnormalities. The possibility that social stimuli act as potent zeitgebers in the arrhythmic R6/2 mice clearly warrants further investigation.

The fact that Modafinil-treated R6/2 mice and their vehicle-treated control mice showed behavior identical to that of the WT mice during the test of rousability was unexpected, because we had previously suggested that progressive motor dysfunction in R6/2 mice contributed to their progressive reluctance to rouse (Pallier et al., 2007). We did additional experiments to test whether or not the steady decline in rousability seen in R6/2 mice when the test was performed 3 times daily was due to altered anxiety levels. However, we found no change in anxiety in either the open-field test or the elevated zero maze. Thus, it seems reasonable to suggest that the progressive deficits in rousability of R6/2 mice stems from “progressive behavioral disengagement” of the mice during testing, and are a mouse correlate of apathy rather than a sign of motor dysfunction. Apathy is a complex syndrome that can be defined as “the quantitative reduction of self-generated voluntary and purposeful behaviors” (Levy and Czernecki, 2006). Thus, apathy can be thought of as a pathology of voluntary action, resulting from dysfunctions occurring at the level of elaboration, execution, and control of goal-directed actions. There is currently no test available to assess apathy in rodents. We suggest that our test of rousability might be useful for measuring apathy because it assesses reduction in self-generated goal-directed actions. However, since there is no other rodent test of apathy available, we have not validated this claim, which remains speculative. Nevertheless, measuring apathy is important because apathy is a significant and common symptom of HD. In HD patients, apathy correlates with the cognitive changes of the disease and increases in severity with illness duration (for references, see Pallier et al., 2007). Apropos, it is interesting to note that the apathy syndrome of a demented and depressed elderly male was successfully treated after 4 weeks of treatment with Modafinil (Padala et al., 2007). In HD patients, Modafinil given as a single dose did not elicit any significant improvements in cognitive function or apathy ratings (Blackwell et al., 2008). Together with the results of Padala et al. (2007), our data add weight to our suggestion that apathy in HD may be tractable.

The ideal drug regime for managing sleep/wake abnormalities in HD is not known. For example, it would be particularly interesting to know if starting the treatment earlier in the disease, well before the appearance of overt symptoms, would offer a better result. The optimal choice of drugs has also not been explored widely. Typically, sleep-inducing drugs are prescribed empirically, and the effect of hypnotic drugs on sleep has not been systematically tested in HD patients. Benzodiazepines are not usually prescribed chronically, because of problems with tolerance, rebound anxiety, and withdrawal when the treatment is stopped (Chouinard, 2004), although Alprazolam has a relatively low dependence potential compared with other benzodiazepines (Jonas and Cohon, 1993). Modafinil, on the other hand, has a proven record of safety and efficacy as a wake-promoting compound. Our results suggest that Modafinil could be used in conjunction with a hypnotic drug, and/or appropriate behavioral intervention, to regularize sleep/wake activity in HD patients. The beneficial effects of Modafinil might allow a reduced need for strong hypnotic drugs. This would be important for long-term management of the sleep/wake disturbance and cognitive decline, where long-term use of sleeping drugs has negative effects (Chouinard, 2004). Moreover, not only could the sleep/wake disturbance and the cognitive dysfunction be treated with a combination treatment, but apathy may also be ameliorated. This, along with the sleep management, would be valuable both to the patients and their caregiver, since apathy and cognitive dysfunction are strongly related to decline in activities of daily living (Hamilton et al., 2003). Normalizing the sleep/wake activity pattern of HD patients might thus help to significantly delay their institutionalization.

4. Experimental procedures

4.1. Animals

All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the United Kingdom 1986 Animals (Scientific Procedures) Act. Mice were taken from colonies of R6/2 mice established in the University of Cambridge. They were housed within a 12/12-h light/dark cycle (lights on at 07:00 A.M. and off at 07:00 P.M.) in a temperature- (21–23°C) and humidity- (55±10%) controlled environment. Dry food, mash and water were available ad libitum. Water bottles were provided with lowered, elongated spouts. Genotyping was performed by PCR from tail snips taken at 3 weeks of age. After genotyping, mice were sorted by genotype and sex into equal numbers in all treatment groups. As no sex differences were observed, data are presented from both sexes pooled together. R6/2 mice had a mean CAG repeat length of 265±1 (n=122) (Laragen, USA).

4.2. Drug treatment

In each cage, half of the mice were drug-treated; the other mice were given the vehicle. Mice were pseudo-randomly assigned to treatment groups. The numbered sequence of assignment was different in each cage, preventing the experimenters (who were also dosing the mice) from memorizing the sequences. In addition, cages were dosed in a
random order every day. Therefore, experimenters were blind to drug treatment groups within genotypes. Mice were treated from 9 to 10 weeks of age until 18–19 weeks. The age at which treatment was started was chosen empirically. We wanted to treat the mice for at least 3 weeks before starting behavioral testing. We started treatment at 9 weeks of age because we wanted to start two-choice swim tank at 12 weeks. We planned to test mice for reversal in the two-choice swim tank. This takes several weeks of training and requires that the mice are capable of swimming throughout the experiment. We wanted the mice to be able to complete the task, so we chose to use R6/2 mice bearing 265 CAG repeats since they do not show an overt phenotype by 12 weeks, the age at which they started training in the two-choice swim tank (Morton et al., 2009). Alprazolam (8-Chloro-1-methyl-6-phenyl-4H-[1,2,4] triazolo[4,3-a][1,4]benzodiazepine) was dissolved in 2.5% methanol in 0.9% saline and administered (1.0 mg/kg, 1 ml/100 g body weight) as a single injection (intraperitoneally) daily between 9:00 A.M. and 10:00 A.M. Modafinil ([diphenylmethyl)sulfinyl]-2 acetamide) was administered (64.0 mg/kg, 0.5 ml/100 g body weight) daily between 6:00 P.M. and 6:30 P.M. by gavage as a 4% corn flour suspension, using a 22-gauge stainless steel gavage needle. Volume of vehicle was equivalent to that used to administer the drugs. Forty five R6/2 mice and 16 WT mice of each sex were used. Mice were given either Alprazolam (n=16 WT and n=16 R6/2), vehicle for Alprazolam (2.5% methanol in 0.9% saline; n=16 WT and n=16 R6/2), Modafinil (n=14 R6/2), vehicle for Modafinil (4% corn flour suspension; n=15 R6/2), Alprazolam and Modafinil (in the morning and evening respectively, as above; n=15 R6/2) or Alprazolam vehicle and Modafinil vehicle (in the morning and evening respectively, as above; n=14 R6/2). We found, in a pilot study (data not shown), that non-injected control mice handled similarly to vehicle-treated mice (that is, held daily by the loose skin at the back of the neck with abdomen upwards, the position used to inject mice intraperitoneally) performed similarly to vehicle-treated controls in the two-choice swim tank. Therefore, non-injected control mice were not included in this study.

4.3. Visual discrimination in the two-choice swim tank

The acquisition of a simple left–right visual discrimination was measured using the two-choice swim tank task (see Pallier et al., 2007). In this task, acquisition is assessed by measuring choice accuracy, a measure that is independent of motor disability (for further discussion, see Lione et al., 1999). Note that in our previous study (Pallier et al., 2007), R6/2 mice treated chronically with Alprazolam were not tested for reversal learning in this task, so direct comparison are not possible.

During each acquisition trial, a transparent escape platform was placed (in a pseudorandom order) at either end of the swim tank, its top surface 0.5 cm below the water level. The position of the platform was cued by an illuminated circle positioned above the platform (an irrelevant black circle being present at the other end of the tank).

For all treatments, mice were tested for acquisition of the task for 12 days, 5 days per week, between 12 and 14 weeks of age. Note that this training protocol was different to that used in our previous study (Pallier et al., 2007), where mice were trained for 7 consecutive days during acquisition. On day 13, the cues were reversed: the platform was moved to the unlit end of the swim tank and its position was cued by the black circle. Mice were tested for reversal acquisition of the task for another 13 days, between 14 and 17 weeks of age. Mice in the Alprazolam alone and Modafinil alone treatment groups had a further 10 days of reversal training. Mice were tested during the second half of the light period.

4.4. Rousability/apathy scoring

The groups of mice that were treated with Alprazolam or with Alprazolam/Modafinil were tested for rousability in response to mild cage disturbance (cage opening) daily during the course of treatment, 3 times per day, as previously described (Pallier et al., 2007). The test was performed 3 times per day to make sure that the mice injected with the hypnotic dose of Alprazolam were still sleeping 1 h after injection, were not drowsy 4 h after injection, just before being tested for behavior, and did not show any sign of residual drowsiness the next morning before injection. The group of mice that was treated with Modafinil was tested for rousability only once a day, in the morning between 9:00 A.M. and 9:30 A.M.

4.5. Body weight

Mice were weighed daily, so that body weights were used to calculate the daily drug doses. Body weights were averaged for each week and the mean body weight was used for data analysis. Body weight data were also analyzed as percentage of body weight loss, taking the weight values from the day before the first day of dosing as the 100% reference.

4.6. Statistical analyses

Repeated-measures analyses of variance (ANOVA) and Student’s t test were used to test statistical significance (Statistica, release 6.0, StatSoft, Inc., Tulsa, USA). For repeated-measures ANOVA, the Huynh–Feldt correction to degrees of freedom was applied to reduce the impact of heterogeneity of variance on the repeated-measures design (assessed by the Mauchly sphericity test), and the corrected probabilities were used. The Bonferroni test was used for multiple independent post-hoc pair-wise comparisons of weight values between the different treatment groups of R6/2 mice. A critical value for significance of p<0.05 was used throughout the study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2009.03.072.
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