Delayed onset and reduced cognitive deficits through pre-conditioning with 3-nitropropionic acid is dependent on gender and CAG length in the R6/2 mouse model of Huntington's disease

Elizabeth A. Skillings & A. Jennifer Morton*

Department of Physiology, Development and Neuroscience, University of Cambridge,

Cambridge, CB2 3DY, United Kingdom

*Address for correspondence:

Prof Jenny Morton

Department of Physiology, Development and Neuroscience,

University of Cambridge

Downing Street

Cambridge CB2 3DY

Telephone +44 1223 334057

Fax +44 1223 334100

Email ajm41@cam.ac.uk

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Abstract

Impairments in energy metabolism are implicated Huntington's disease (HD) pathogenesis. Reduced levels of the mitochondrial enzyme succinate dehydrogenase (SDH), the main element of complex II, are observed post mortem in the brains of HD patients, and energy metabolism defects have been identified in both presymptomatic and symptomatic HD patients. Chemical preconditioning with 3-nitropropionic acid (3-NP), an irreversible inhibitor of SDH, has been shown to increase tolerance against experimental hypoxia in both heart and brain. Here we studied the effect of chronic preconditioning using low doses of 3-NP (24 mg/kg), administered via the drinking water, on cognition function and phenotype progression in the R6/2 mouse model of HD. R6/2 mice carrying CAG repeat lengths of either 250 or 400 repeats were used. Both are transgenic fragment models, with 250CAG mice having a more rapid disease progression than 400CAG mice. After 3-NP treatment there were significant improvements in all aspects of the behavioural phenotype, apart from body weight, with timing and magnitude of improvements dependent on both CAG repeat length and sex. Specifically, a delay in the deterioration of general health (as shown by delayed onset of glycosuria and increased survival) was seen in both male and female 400CAG mice and in female 250CAG mice and was consistent with improved appearance of 3-NP treated R6/2 mice. Male 250CAG mice showed improvements but these were short term, and 3-NP treatment eventually had deleterious effects on their survival rate. When cognitive performance of 250CAG mice was assessed using a two-choice discrimination touchscreen task, we found that female mice showed significant improvements. Together, our results support the idea that energy metabolism contributes to the pathogenesis of HD, and suggest that improving energy deficits might be useful therapeutically.

Introduction

Huntington's disease (HD) is an autosomal dominant neurological disease, characterised by early neurodegeneration of striatum and cortex [1]. Clinical signs and symptoms of HD include chorea, dystonia, psychiatric disturbance and cognitive decline [2]. While the mechanisms underlying the neurodegeneration remain unclear, defects in energy metabolism have been identified in both presymptomatic [3-4] and symptomatic [5] HD patients, as well as in post mortem HD brain [6]. Furthermore, patients in advanced stages of HD experience unexplained weight loss (cachexia) despite an increased calorie intake [7-8]. Cachexia has also been well documented in the R6/2 mouse model of HD [9-12].

Studies of energy metabolism in humans reveal a progressive decline in mitochondrial enzyme activity with normal aging [13-14]. Energy metabolism has been the focus of research in HD for many years and various hypotheses have been put forward to explain its possible role in the pathogenesis of HD. Beal first proposed a role for mitochondrial defects in striatal vulnerability [15-17]. An earlier hypothesis constructed to explain HD striatal pathology was the 'excitotoxicity hypothesis'[18-19] [20-24], whereby increased sensitivity and/or prolonged or excessive exposure to glutamate, the principal excitatory neurotransmitter, results in striatal excitotoxicity [25]. Energy deficits exacerbate excitotoxicity [26-27] and the current thinking is that multiple mechanisms, including excitotoxicity, energy deficit, oxidative stress, inflammatory processes, and protein aggregation, are interlinked, and together trigger the neurodegenerative process [28-29].

Succinate dehydrogenase (SDH), the main element of Complex II, is an enzyme involved in both the tricarboxylic acid cycle (TCA) and the electron transport chain. Reduced levels of SDH have been observed post mortem in the brains of HD patients compared to normal controls [30]. Similar deficiencies have been identified in the R6/2 mouse. For example, whole brain protein extracts show transient changes in the expression of Ip (a subunit of Complex II) between 2 and 12 weeks of age [31], while high-resolution respirometric (HRR) studies reveal reduced levels of Complex II in the striatum at 12 weeks of age [32]. 3-nitropropionic acid (3-NP) is a specific and irreversible inhibitor

of SDH [33-34] and systemic administration to rodents eventually causes spontaneous bilateral striatal lesions, with sparing of NADPH diaphoase interneurons and reactive gliosis [16, 35-37]. Indeed, before the development of transgenic HD mouse models 3-NP toxicity was widely used as model of HD in rats [16, 38] and mice [39-40].

In this study, 3-NP was not used as a toxin to induce lesions. Rather, we used 3-NP in a therapeutic strategy known as 'preconditioning' [41]. The theory behind preconditioning, first described by in 1964 [42], is that administration of a toxic substance at a sub-toxic level induces a response that is protective, or at least limits the degree of damage caused by subsequent presentation of a similar or stronger dose of the toxin. Chemical preconditioning with 3-NP has been used to show increased tolerance against hypoxia in both heart and brain [43-47], although these studies used only acute exposure periods to the preconditioner. Nevertheless, these short periods of preconditioning were able to confer a significant period of defence (an effect known as the first window of protection) that lasted for 1-2 hours post-exposure [48-49]. There is also evidence for a second window of protection that is a delayed, less vigorous response, which occurs 24 hours post-exposure [50]. We wondered if preconditioning would have any effect on deterioration of phenotype of HD mice. We already have evidence that there is endogenous protection against disease that derives from genetic mutation, since young R6/2 mice are less vulnerable than WT mice are to 3-NP [41]. Furthermore, excitotoxins such as kainic acid [28] and quinolinic acid [51] are less effective in juvenile R6/2 mice than they are in WT mice. We have suggested that this is due to autoprotection against endogenous toxicity of the mutant huntingtin protein, a product of the HD transgene. In the present study, we sought to investigate the effect of chronic preconditioning with 3-NP as a treatment strategy, to establish whether prolonged treatment with low dose 3-NP could delay phenotype progression and/or improve cognition function in the R6/2 mouse model of HD.

Methods

Animals

All components of this study were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and with the approval of the University of Cambridge Animal Welfare and Ethical Review Board. Lighting was controlled on a 12h light:12h dark cycle, with the light period starting at 07:30h. Behavioural testing was conducted during the light phase. The housing facility temperature was maintained at 21–23°C and the relative humidity was controlled (55 ± 10%). Mice had *ad libitum* access to water via lowered waterspouts and standard dry laboratory food with access to nesting materials and a Perspex house in the home cage. In addition, mice were given a daily supplementary feed of 'mash' (pellets soaked in water overnight), one pellet per mouse, unless otherwise stated. A total of 252 mice (for details see below) were used for this study. Mice were taken from colonies of R6/2 mice established in the University of Cambridge and maintained by backcrossing onto CBA x C57BL6 F1 female mice. Genotyping was performed by PCR from tail snips taken at 3 weeks of age. CAG repeat lengths were measured by Laragen (USA) and determined using Genemapper software. R6/2 mice had CAG lengths of either 250 (255 ± 1; n=84) or 400 (397 ± 1; n=43) repeats.

Experimental Groups

Mice were age-matched and housed in single sex, single genotype cages of 10-12 mice. Single genotype groups were used to remove any potential increases in appearance or survival which may result from WT mice acting as 'nursemaids' to the transgenic mice. We have shown previously that increased activity of WT mice, such as that which occurs at mash feeding times, encourages R6/2 mice housed with them to rouse and feed and modifies both their behaviour and survival [52]. Half the mice received low dose 3-NP in their drinking water, the other half received water only, and acted as vehicle controls. Two experiments were performed. In the first, 250CAG (n=20 male: 10 3-NP treated, 10 control; n=20 female: 10 3-NP treated, 10 control), 400CAG (n=22 male: 11 3-NP treated, 11 control; n=21 female: 10 3-NP treated, 11 control) and age matched WT littermate (n=41 male: 20 3-NP treated, 21 control; n=40 female: 20 3-NP treated, 20 control) mice were used for behavioural phenotype assessment (SHIRPA, glycosuria testing, body weight and survival analysis) as outlined

below. In the second, 250CAG (n=20 male; n=24 female) and age matched WT littermates (n=20 male; n=24 female) mice underwent cognitive assessment using the touchscreen system.

Drug Administration

For one week prior to drug administration, water bottles containing tap water were weighed daily, to determine the average daily water intake per group for initial drug dose calculations. The drug was calculated on a per-cage, rather than per-mouse basis. Once the drug was introduced to the water bottles there was no decrease in water intake, suggesting that there were no issues with palatability of water containing 3-NP. 3-NP treated mice received 3-NP in their drinking water from 5 weeks of age (250CAG cognitive assessment groups), 8 weeks of age (250CAG phenotype assessment groups) or 15 weeks of age (400CAG phenotype assessment groups). The cognitive assessment group began treatment earlier to allow treatment to take place prior to cognitive testing which began at 8 weeks of age. The remaining mice received tap water and served as controls. The target dose of 3-NP was 24 mg/kg daily. This was administered as a rising concentration. Initial dosing began at12 mg/kg, until the target dose of 24 mg/kg was reached over the course of a week (dose was increased in 3 mg/kg increments every second day). After the first week, all water bottles were refreshed twice weekly, at the same time of day. Water bottles were weighed daily (at the same time each day) to confirm volume intake, and mice were weighed twice weekly to recalculate the dose rates. The average water intake per cage, from the previous seven days, was used to calculate the drug dose for the next dosing period.

Experiment 1 - Phenotype Assessment

SHIRPA

We used a modified version of the SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment (SHIRPA) protocol (SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Center and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's; Royal London Hospital, St Bartholomew's and the Royal London School of Medicine). The full modified protocol used for SHIRPA testing is outlined in Glynn et al [53]. In brief, a mouse

was removed from the home cage to be individually evaluated in a clean cage. We visually assessed the mice for a number of phenotypic elements, including piloerection, lordokyphosis, parapebral closure, fore and hindlimb clasping, tremor and righting reflex. 250CAG mice deteriorate more rapidly than 400CAG mice [12] so 250CAG mice were tested at two week intervals from 8 weeks of age and 400CAG mice at 4 week intervals from 15 weeks of age until death. However, only data collected up to 20 weeks of age for 250CAG mice and 44 weeks of age for 400CAG mice (a time point where all mice were still alive) were included in statistical analysis. Data were quantified using a binary system where a 'normal' behaviour received a score of 0 and an 'abnormal' behaviour received a score of 1and a cumulative score determined for each mouse at each timepoint. Higher scores indicate a greater degree of abnormality.

Portrait photographs

Portrait photographs were taken of all mice at 20 (250CAG mice) and 40 (400CAG mice) weeks of age from which the angle of spinal curvature was measured as an indication of the degree of lordokyphosis present.

Glycosuria testing

Urine samples were collected weekly from mice and tested using Diastix reagent sticks (Bayer PLC, Berks, UK), for detection of glycosuria. The presence of a glycosuria reading ≥ 111 mmol/L was regarded as indicative of diabetic status.

Body weights and survival

Body weights were recorded twice weekly from 8 (250CAG groups) or 15 (400CAG groups) weeks of age, until the last R6/2 mouse had died or was killed for humane reasons. However, only data collected up to 20 weeks of age for 250CAG mice and 45 weeks of age for 400CAG mice (a time point where all mice were still alive) were included in statistical analysis. Age of death was recorded for all R6/2 mice. Mice were killed when they reached a defined end point (if they were moribund, lacked a righting reflex, or failed to respond to 10 seconds of gentle stimulation).

Experiment 2 – Cognitive Assessment

Behavioural testing was carried out in 12 automated touchscreen testing chambers. The task used for testing is similar to that described previously [54]. Mice were trained to use the touchscreen at 5 weeks of age and began discrimination training at 8 weeks of age. They were given 14 daily sessions of 30 two-choice discrimination trials. After this, the stimuli were reversed for a further 14 sessions with the new stimulus-reward contingency. Apparatus consisted of an infrared touchscreen (Craft Data Ltd., Bucks, UK) and a standard modular testing chamber housed within a sound-attenuating box (Med Associates Inc., Vermont, USA) fitted with a 28 volt DC fan for ventilation and masking of exterior noise. The inner operant chamber (21.6 x 17.8 x 12.7 cm; Med Associates Inc., Vermont, USA) consisted of a metal frame with clear Perspex walls and a stainless steel grid floor. A 3W houselight, pellet receptacle (magazine) and a tone generator (Med Associates Inc., Vermont, USA) were fitted to the rear wall. The magazine was illuminated by a 3W light bulb and fitted with photocell head entry detectors to detect the presence of a mouse retrieving a reward pellet. Pellets were dispensed by a 14mg pellet dispenser situated outside the testing chamber. Stimuli were presented on an infrared touchscreen located at the opposite end of the chamber. A red Perspex 'mask', containing two response windows behind which the stimuli were displayed, was positioned in front of the touchscreen. Windows were positioned 1.6 cm above the chamber floor, to prevent the mouse triggering the touchscreen with its tail. To maintain motivation to work for reward pellets, starting from three days prior to testing, and continuously throughout cognitive testing, mice were placed on a food-restricted diet, such that their body weight was no less than 85% of the free-feeding body weight of a standard R6/2 mouse. Mice were fed mash within one hour of completing testing.

Statistical analysis

Statistical analyses were performed using Statsoft Statistica v12 software (Statsoft, Tulsa, Oklahoma, USA) and Prism 5 (GraphPad Software Inc., San Diego, USA). SHIRPA abnormality scores were analysed using a repeated measures ANOVA. Although SHIRPA and body weight data were collected until end stage, only data up to 20 weeks for 250CAG groups and up to 45 weeks for 400CAG groups is presented and analysed since after this time a number of mice had died or been killed and could therefore not be included in longitudinal analysis. For analysis of touchscreen testing

we used repeated measures ANOVA (factors: treatment and genotype). Bonferroni's post-hoc test was used to determine differences, when significant group effects were identified. Significance levels were set at $p<0.05$ for all analyses.

Results

3-NP treatment delays progression of the HD phenotype

Both groups of WT control mice had a mean SHIRPA index of 1 at each data collection point, irrespective of treatment. This reflects their 'normal' phenotype (smooth coat, standard gait etc.). Delays in phenotype progression, as indicated by a lower SHIRPA abnormality index, were seen in 3-NP treated 250CAG male and female mice (Figure 1A, B) and 400CAG repeat female mice (Figure 2B) compared to R6/2 untreated control groups. In the male 250CAG 3-NP treated mice there was a significant delay in phenotype development from 14 to 18 weeks of age, as reflected in their higher SHIRPA abnormality index ($F_{(6, 204)}=3.3556$, $p<0.01$; Figure 1A), but this improvement was lost by 20 weeks. A similar effect was also seen in the female 250CAG 3-NP treated mice, with a significant HD phenotype evident from 18 weeks of age compared to R6/2 vehicle treated control mice ($F_{(6, 216)}=16.129$, $p<0.001$; Figure 1B). In the 400CAG groups, there was a significant beneficial effect of 3-NP treatment on SHIRPA index in female mice from 36 weeks onwards (Figure 2B; $F_{(7, 238)}=14.553$, $p<0.001$). These delays in phenotype progression were consistent with improved appearance of 3-NP treated R6/2 mice (Figure 3). For example, female 3-NP treated 400CAG mice in particular show a glossy coat and bright eyes, compared to vehicle treated counterparts (Figure 3E,F). We also saw a reduction in the degree of lordokyphosis present in the male 250CAG R6/2 mice at 20 weeks of age ($F_{(1,12)}=19.275$, $p<0.001$; Figure 3C,D). There was a similar effect seen in male 400CAG mice ($F_{(1,16)}=4.8924$, $p<0.05$; Figure 3G,H) at 45 weeks of age, despite the lack of improvement seen in the SHIRPA index.

3-NP treatment reduces weight gain in WT mice

All R6/2 mice had lower body weights than their WT counterparts throughout the study. The body weight increase (as a percentage change from baseline) of WT mice in those groups receiving 3-NP

from 8 weeks of age was significantly lower than that of WT mice in the vehicle treated group in both male ($F_{(1, 35)}=10.257$, $p<0.01$) and female mice ($F_{(1, 33)}=7.3986$, $p<0.05$) (Figure 1 C,D). This effect was not present in WT mice in the 400CAG group treated from 15 weeks of age.

3-NP treatment delays the onset of glycosuria

R6/2 250CAG mice typically develop glycosuria between 9 and 14 weeks of age [55]. While glycosuria in 400CAG mice has not been reported previously, here, all R6/2 mice developed glycosuria during the course of treatment. No WT mice developed glycosuria at any stage of the experiment, regardless of treatment group. Delays in the onset of glycosuria were seen in 250CAG repeat 3-NP treated female mice ($X^2=5.353$; $p<0.05$; Figure 1F). Mean age of onset for mice in the female control groups was 15.6 weeks compared to that of 3-NP treated female mice who developed glycosuria at 18.7 weeks. There was no significant effect of 3-NP treatment in male 250CAG mice, with mean age of onset being 16 weeks for 3-NP treated mice and 17.8 weeks for vehicle control mice. In 400CAG repeat mice, 3-NP treatment resulted in a significant delay in the age of onset of glycosuria in both male ($X^2=8.825$; $p<0.01$) and female ($X^2=5.863$; $p<0.05$) mice (Figure 2 E, F). Mean age of onset in control groups was 30.4 weeks (males) and 37.6 weeks (females) compared to 3-NP treated mice that developed glycosuria much later at 36.2 weeks (males) and 48 weeks (females).

3-NP treatment improves survival in R6/2 mice

3-NP treated male 250CAG mice died significantly earlier than untreated control groups ($X^2=19.95$; $p<0.0001$; Figure 1, G). However, 3-NP treatment improved survival in female 250CAG repeat mice ($X^2= 10.44$; $p<0.01$) (Figure 1, H) and male ($X^2=8.729$; $p<0.01$) and female ($X^2=7.049$; $p<0.01$) 400CAG repeat mice (Figure 2G, H). No WT mice died during the course of the study.

3-NP treatment has minor beneficial effects on performance in the touchscreen

R6/2 mice show progressive deficits in touchscreen performance dependent upon the age at which testing begins [54]. No significant improvements were seen in male 250CAG mice for any of the measures assessed (Figure 4D,E,F; 6B). Improvements in performance were seen in 3-NP treated

female 250CAG mice during the acquisition phase of testing (Figure 5D,E,F). Significant improvements were found in three measures of performance; total correction trials per session, time to complete a session and the number of sessions required to reach criterion. Female 3-NP treated R6/2 mice required significantly fewer correction trials per daily session compared to all other groups ($F_{(1,42)=}6.7764$, $p<0.05$; Figure 5F) and took significantly less time than their vehicle-treated counterparts to complete each daily session of 30 trials during the acquisition phase of training ($F_{(1,42)}=8.8327$, $p<0.001$; Figure 4H). The number of sessions required to reach criterion ($\geq 70\%$) by 3-NP treated R6/2 female mice was not significantly different to that required by WT mice, regardless of treatment group (Figure 6B). It was also not significantly different from vehicle-treated female R6/2 mice, although they needed significantly more sessions that WT mice to reach criterion ($F_{(1,42)}=4.8938$, $p<0.05$; Figure 6B).

Discussion

In this study, we found that preconditioning with 3-NP delays the onset of phenotype in male and female R6/2 mice with 250 repeats. It also improves cognitive performance in the touchscreen, delays the onset of glycosuria and increases survival in female 250CAG R6/2 mice. 3-NP preconditioning also delays onset of glycosuria and increases survival in both male and female 400CAG mice.

Deficiencies in energy metabolism have long been a focus of HD research and various mechanisms have been put forward to explain the role that such deficiencies might play in the progression of HD. It is possible that in HD patients, while a mild energy metabolism impairment may always be present, deleterious effects which might cause neuronal energy production to fall below a critical level, may not become obvious until potentiated by the effects of reduced mitochondrial electron transport enzyme activity seen in normal aging [13-14]. Metabolic deficits therefore, are often not measurable until the disease has progressed to the later stages. Here we used 3-NP preconditioning using R6/2 mouse models to test the idea that there was a capacity for improvements in energy metabolism and that this would manifest as phenotypic improvements in our mice. Our working hypothesis was that mild inhibition of SDH caused by low level 3-NP administration functionally upregulated SDH, so

that levels are increased by the time that disease-related impairments in energy metabolism are seen. This may confer protection to striatal neurons through increased stabilisation of the SDH complex, as suggested by Benchoua et al [56] who restored SDH levels in rat striatal neurons using lentivirus-mediated gene overexpression. They showed that the overexpression of either the Ip or Fp subunit restored complex II levels and blocked mitochondrial dysfunction and striatal cell death. Note that we did not measure SDH levels, since we have shown previously that the available assays are not sensitive enough to pick up small changes [41, 57] and we also took our mice to end stage to maximize the behavioural data available from the study. In future studies it would be valuable to determine whether or not, at the time when the biggest changes in behaviour occurred, there were changes in SDH activity, transcriptional and or protein levels. This would best be measured at a much earlier age than was possible in our study.

WT mice gain weight when kept on an *ad lib* feeding regime. If 3-NP treatment is modifying energy metabolism then a slower degree of weight gain in treated WT groups compared to untreated controls would be expected. Interestingly, a similar effect was not seen in the R6/2 mice receiving 3-NP, even though weight loss is pronounced in these mice one they become symptomatic. This may be a result of the increased metabolism already known to exist in these mice [11].

It is possible that the effects of 3-NP we found here are not mediated via direct effects on the CNS but rather, they may be mediated via cardiovascular improvements. Although HD is unequivocally a neurological disease, cardiac dysfunction is the second most common cause of death in HD patients (for review, see Zielonka et al [58]), and has also been identified as a potential factor in the deterioration of the R6/2 mouse [59-61]. We have suggested previously that cardiac dysfunction plays a role in the development of the R6/2 phenotype, with hearts of R6/2 mice having significant differences in terms of function and morphology compared to WT mice, which increase in severity in line with disease development [60].Gabrielson et al [62] studied the effect of 3-NP toxicity on the cardiac function of four strains of mice (BALB/c, C57BL/6, FVB/n and 129SvEMS) and found that

significant levels of cardiac toxicity accompanied the neurotoxicity in each. They suggest that the inhibition of SDH in heart mitochondria contributes to the cause of death in 3-NP poisoning in both acute and subacute/chronic studies in mice. Studies investigating chemical preconditioning of rat heart with 3-NP would support this theory. Hu et al [63] found that chemical preconditioning with 3-NP has cardioprotective effects against ischemia-reperfusion injury in rat heart, with 3-NP treated animals recovering significantly better following ischemia-reperfusion injury. Basgut et al [64] reported similar findings, with 3-NP preconditioning significantly reducing infarct size, creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH) levels, ventricular tachycardia (VT) compared to untreated controls. If, as suggested earlier, we are restoring SDH levels by preconditioning with 3-NP, then it may be that we are also reducing (or at least delaying) cardiac dysfunction in our mice, resulting in improved survival rates in the majority of our mice. No studies have directly evaluated sex differences in the age of onset of cardiac dysfunction in R6/2 mice, although it is reasonable to assume that onset of cardiac dysfunction may be delayed in female mice, in line with other aspects of the HD phenotype. As we did not begin 3-NP dosing until 8 weeks of age (by which age cardiac dysfunction was already identified in male mice in the Mimh et al study [59]) this may explain the lack of improvement in survival of male 250CAG R6/2 mice. Indeed, it is possible that 3-NP treatment exacerbated HD related cardiac deficits in the male mice resulting in the earlier deaths seen in this study.

Improvements seen in female R6/2 mice were generally larger than those seen in male R6/2 mice in the majority of aspects of this study, in both 250CAG and 400CAG mice, the only exception being lordokyphosis. This is consistent with previous data from our laboratory showing that behavioural and cognitive deficits consistently appear earlier in male R6/2 mice than they do in female R6/2 mice and that male R6/2 mice die earlier [65-66]. These sex differences are consistent with a previous study where Nishino et al [67] found gender and sex-hormone dependent differences in the vulnerability to 3-NP intoxication in rats. They suggested that not only does oestrogen protect against vulnerability to chemical hypoxia by 3-NP, but also that testosterone exacerbates the vulnerability. This suggestion is supported by data from Mogami et al [68] who found that 17b-estradiol (Tamoxifen) conferred cytoprotective effects to brain endothelial cells under 3-NP stress. It

is possible that the testosterone-induced exacerbation of 3-NP intoxication is strong enough to overcome the beneficial effects conferred by the low-dose 3-NP regime, hence the lack of improvements seen in the SHIRPA testing of 400CAG male mice. This effect may not have been seen in the 250CAG male mice because their testosterone levels drop [69]. Another possible reason is that we did not start treatment of 250CAG repeat mice in the behavioural experiments until they had reached full maturity at 8 weeks of age. By this age, although not overtly manifest, signs of neurological abnormality are already appearing [9, 70-72]. It is possible that had we started treatment of mice earlier, greater improvements would be seen in the R6/2 male mice. Note that it is possible that because the 3-NP was administered in the drinking water, some individuals may have had a marginally greater drug intake than others. This may explain the variability of treatment success seen in some of the testing measures. The dosing method should be considered carefully if preconditioning strategy is investigated in future studies. Controlled delivery by a minipump would eliminate the variability, although it would not be possible to alter the drug dose.

Conclusion

Preconditioning with 3-NP delays the onset of the overt phenotype (male and female 250CAG; female 400CAG). It also delays onset of glycosuria (female 250CAG; male and female 400CAG), improves cognitive performance (female 250CAG) and prolongs survival (female 250CAG; male and female 400CAG). The slowing of phenotype progression supports the idea that regulation of energy metabolism is a significant therapeutic target for development of drug treatments for HD. While chemical preconditioning has been widely studied in heart and brain, the potential of using preconditioning for the treatment of neurodegenerative disease remains largely uninvestigated. It is clear from the improvements shown here that the potential of preconditioning as a therapy should not be overlooked, and should be investigated in more detail in HD animal models. Caution, however, must be exercised when extrapolating potential therapeutic strategies from mice to humans with HD. However promising the preconditioning approach is, it remains that 3-NP is a powerful mitochondrial

toxin whose effects are very variable in humans. The wrong dose could cause brain lesions [73], rather than improve neurological function.

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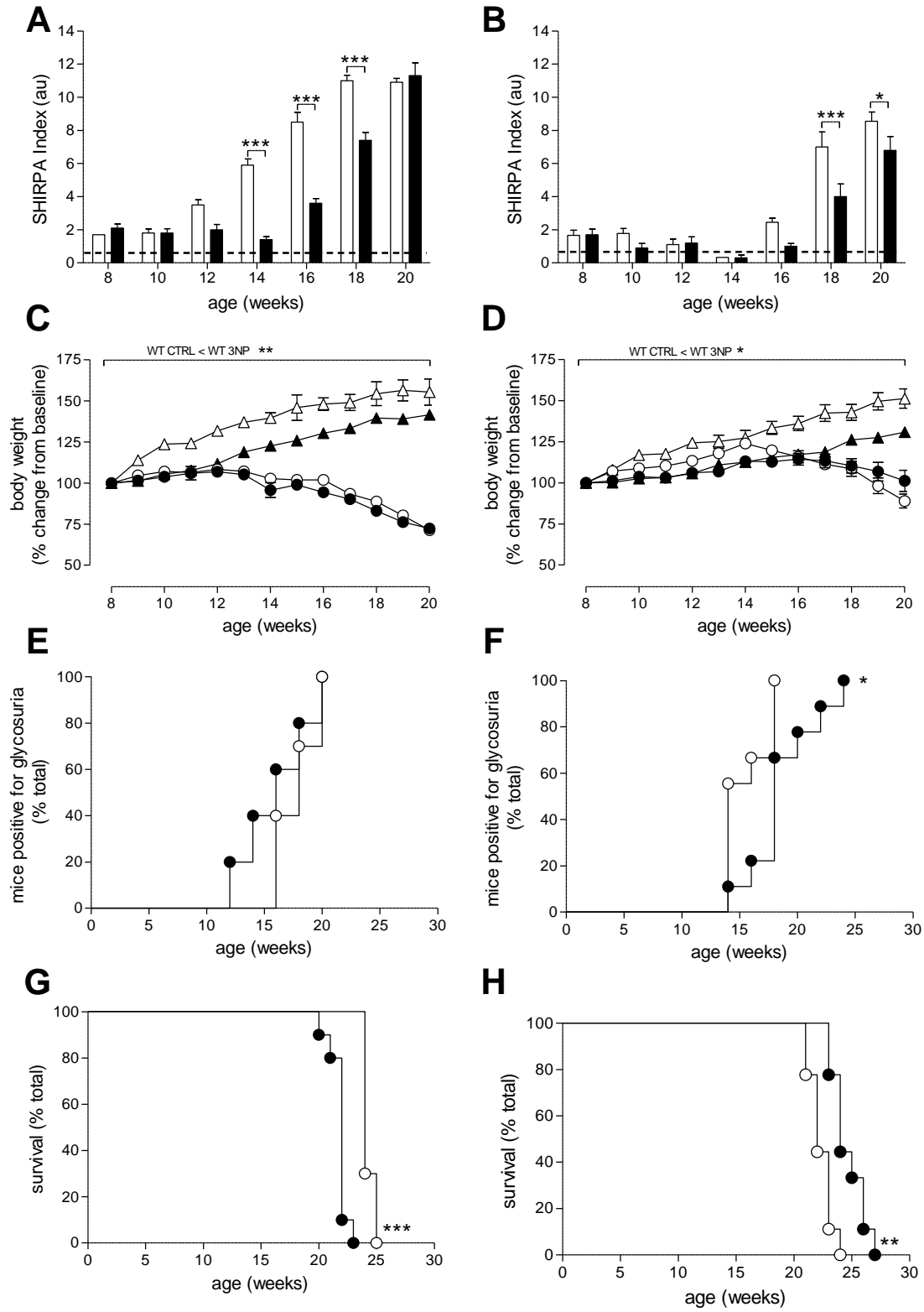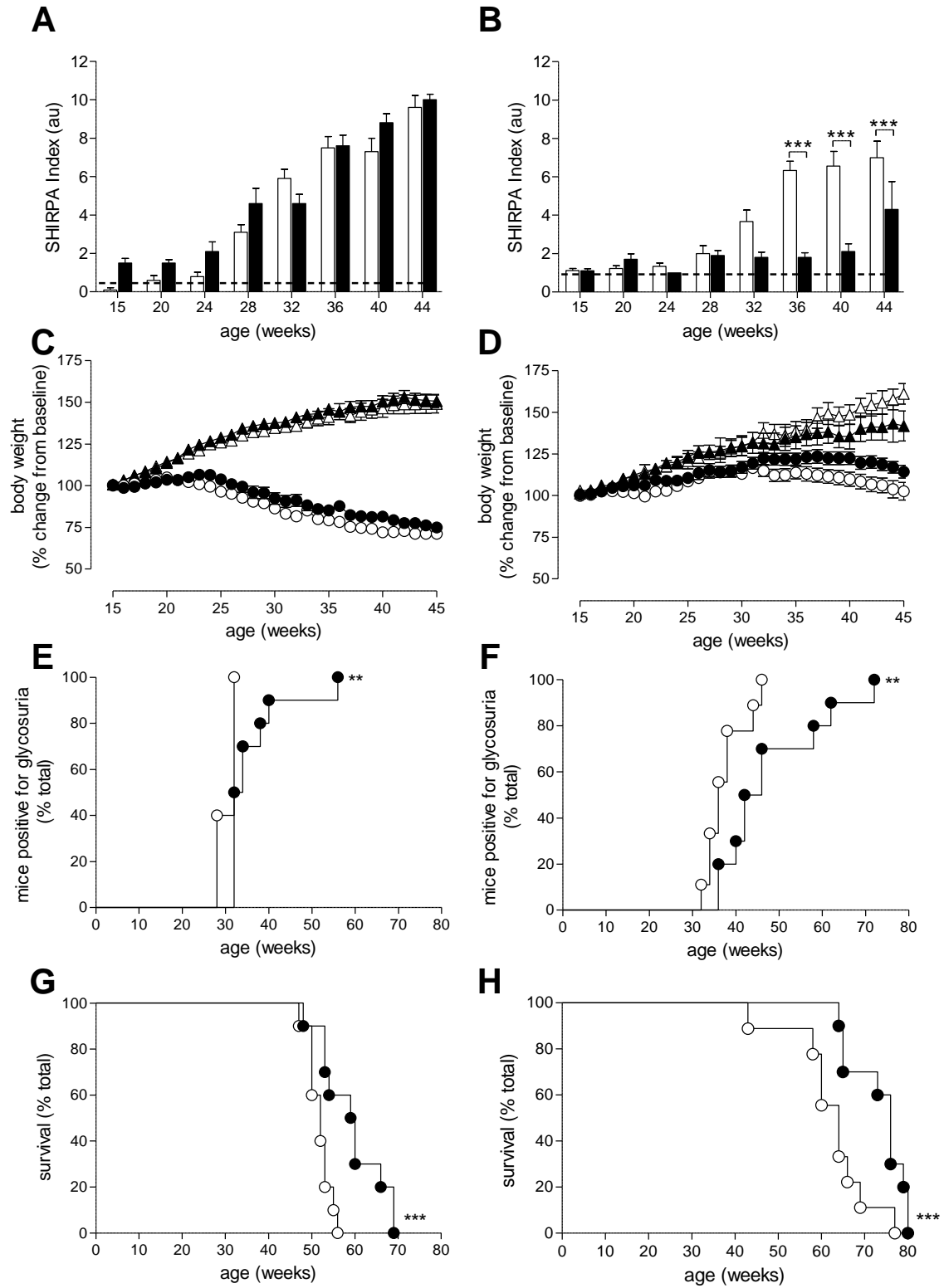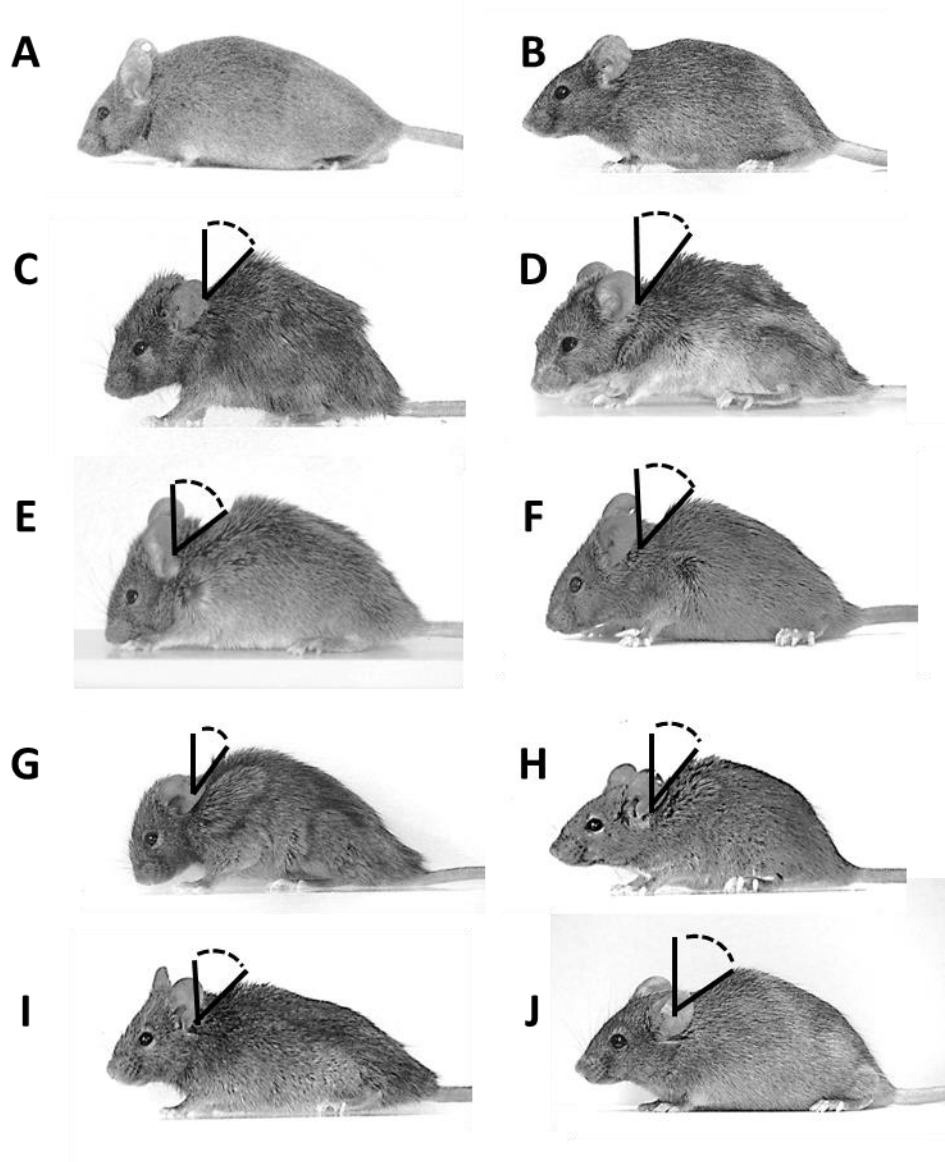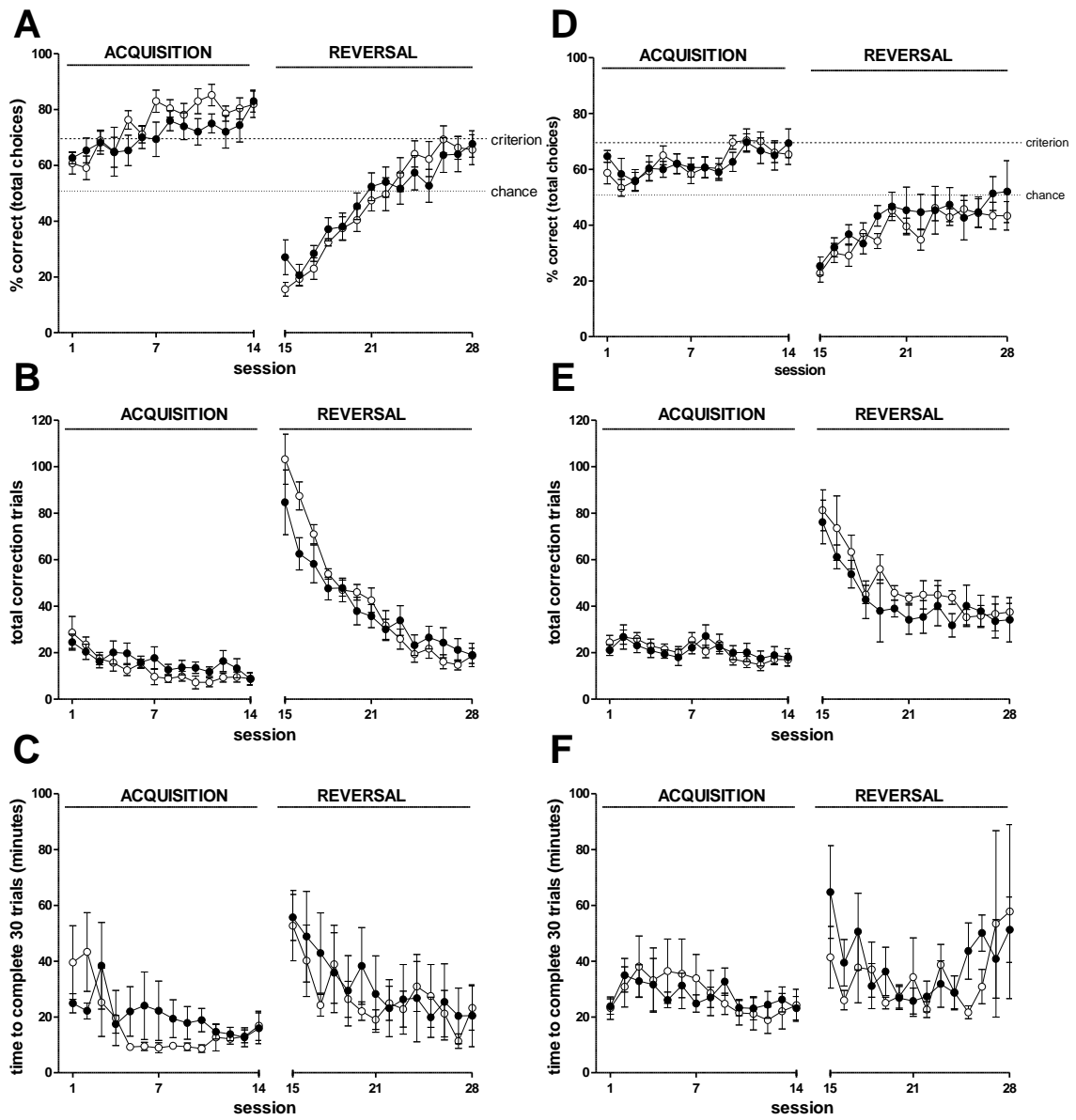
Figure 1

Figure 2
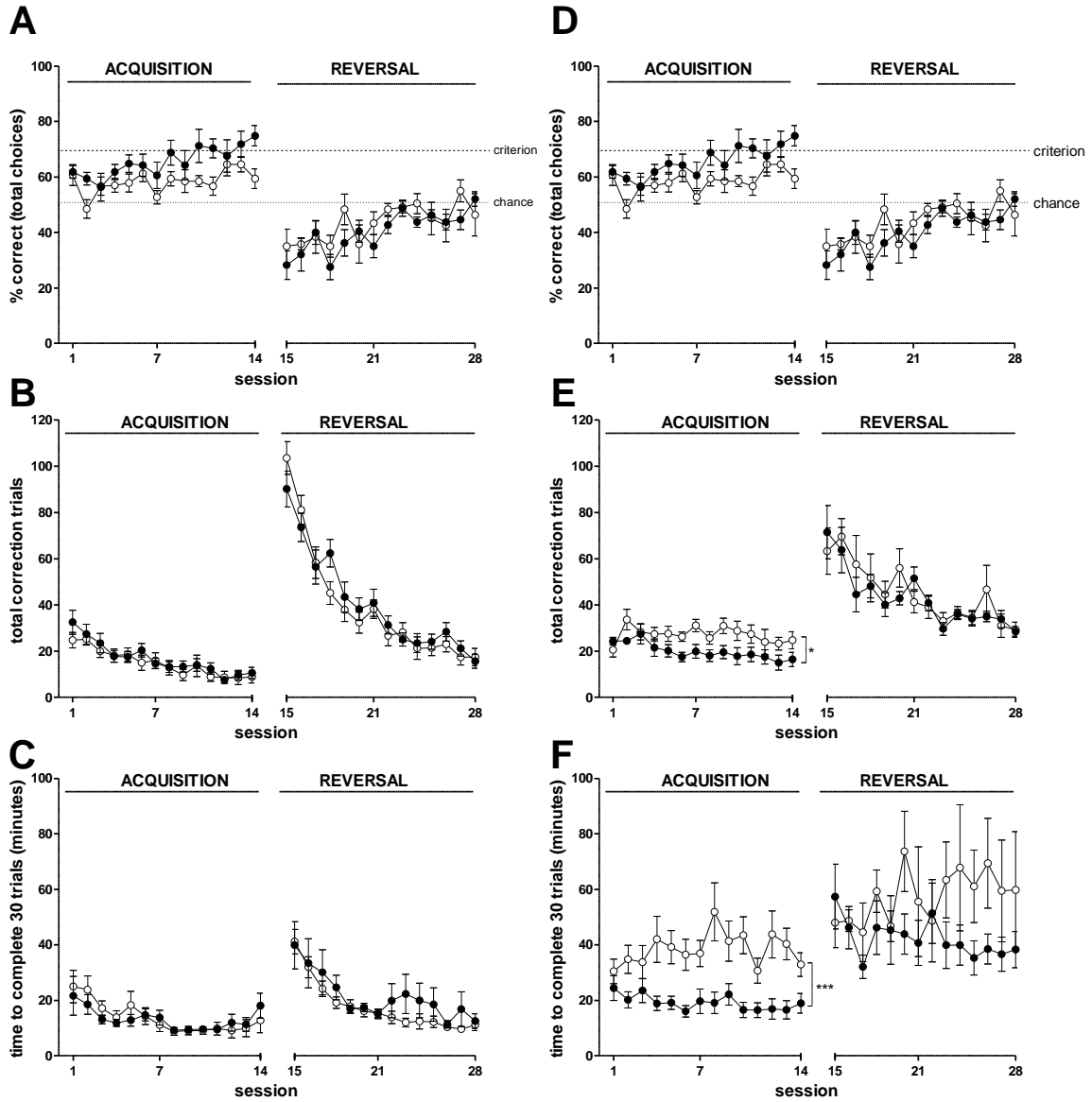
Figure 3

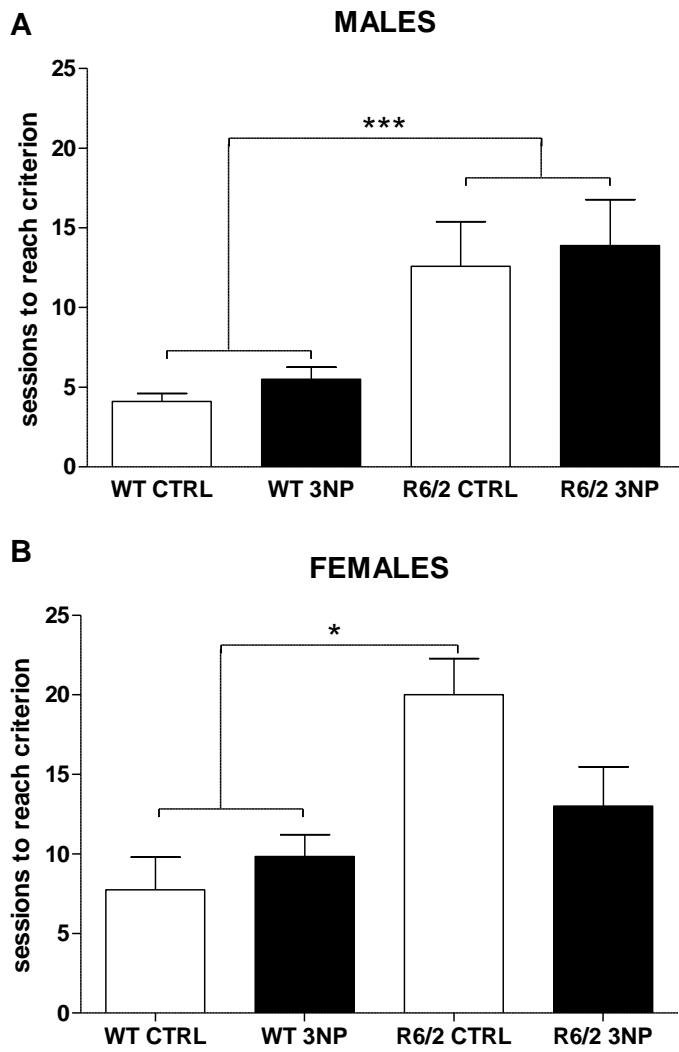Figure 4

Figure 5

A **MALES**

B **FEMALES**

Figure 6

Figure 1: Phenotype assessment of 250CAG mice 3-NP preconditioning delayed onset of phenotype in R6/2 male (A, C, E, G) and female (B, D, F, H) mice. Data shown are mean SHIRPA scores (A,B); body weight changes (C,D); onset of glycosuria (E,F) and survival (G,H). For SHIRPA (A,B) and body weight (C,D) analysis, only data up to 20 weeks was analysed as after this time some mice had died or been killed. N numbers at 20 weeks were 10 male vehicle, 10 female vehicle, 10 male 3-NP treated, 10 female 3-NP treated WT and 10 male vehicle, 10 female vehicle, 10 male 3-NP treated, 10 female 3-NP treated R6/2 250 mice. Data in A-F are mean ± SEM. * indicates differences between treated vs untreated groups *p<0.05; **p<0.01; ***p<0.001. Where error bars are not visible, they are obscured by the symbols. Dotted line in A and B indicates the level of WT score. In A & B filled columns represent R6/2 3-NP treated mice, open columns represent R6/2 control mice; In C & D filled triangles represent WT 3-NP treated mice, open triangles represent WT control mice; In E, F, G & H filled circles represent R6/2 3-NP treated mice and open circles represent R6/2 control mice.

Figure 2: Phenotype assessment of 400CAG mice 3-NP preconditioning delayed onset of phenotype in R6/2 male (A, C, E, G) and female (B, D, F, H) mice. Data show SHIRPA (A,B); body weight changes (C,D); onset of glycosuria (E,F) and survival (G,H). For SHIRPA (A,B) and body weight (C,D) analysis, only data up to 45 weeks was analysed, as after this time some mice had died or been killed. N numbers at 45 weeks were 11 male vehicle, 10 female vehicle, 11 male 3-NP treated, 10 female 3-NP treated WT and 11 male vehicle, 11 female vehicle, 11 male 3-NP treated, 10 female 3-NP treated R6/2 250 mice. Data in A-F are mean ± SEM. * indicates differences between treated vs untreated groups *p<0.05; **p<0.01; ***p<0.001. Where error bars are not visible, they are obscured by the symbols. Dotted line in A and B indicates the level of WT score. In A & B filled columns represent R6/2 3-NP treated mice, open columns represent R6/2 control mice; In C & D filled triangles represent WT 3-NP treated mice, open triangles represent WT control mice; In E, F, G & H filled circles represent R6/2 3-NP treated mice and open circles represent R6/2 control mice.

Figure 3: Representative photographs of late stage R6/2 mice showing angles of spinal curvature

End stage is 20-25 weeks for 250CAG mice and >45 weeks for 400CAG mice. Portraits shown in A, C, E, G and I are: male WT (A), 250CAG control (C), 250CAG 3-NP treated (E), 400CAG control (G) and 400CAG 3-NP treated (I) mice. Portraits shown in B, D, F, H and J are: female WT (B), 250CAG control (D), 250CAG 3-NP treated (F), 400CAG control (H) and 400CAG 3-NP treated (J) mice. Line indicates the angle of spinal curvature.

Figure 4: Cognitive testing of male 250CAG mice in the touchscreen Data show the acquisition and subsequent reversal of a two-choice discrimination task for male WT (A, B, C) and R6/2 (D, E, F) mice. Performance measures shown are percent correct (A,D); total number of correction trials (B,E), number of sessions to criterion (C,F) and time taken to complete 30 trials (D,H). Data points are mean ± SEM. For all panels filled circles (•) represent 3-NP treated mice and open circles (o) represent control mice.

Figure 5: Cognitive testing of female 250CAG mice in the touchscreen Data show the acquisition and subsequent reversal of a two-choice discrimination task for female WT (A, B, C) and R6/2 (D, E, F) mice. Performance measures shown are percent correct (A,D); total number of correction trials (B,E), number of sessions to criterion (C,F) and time taken to complete 30 trials (D,H). Data are mean ± SEM. * indicates differences between treated vs untreated groups *$p < 0.05$; ***$p < 0.001$. For all panels filled circles (•) represent 3-NP treated mice and open circles (o) represent control mice.

Figure 6: Cognitive testing of male and female 250CAG mice in the touchscreen The number of sessions needed to reach criterion for Male (A) and female (B) R6/2 mice are shown in control (open columns) and 3-NP treated (closed columns) mice. Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.