Impulsive choices in mice lacking imprinted Nesp55

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ABSTRACT

Genomic imprinting is the process whereby germline epigenetic events lead to parent-of-origin specific monallelic expression of a number of key mammalian genes. The imprinted gene *Nesp* is expressed from the maternal allele only and encodes for Nesp55 protein. In the brain Nesp55 is found predominately in discrete areas of the hypothalamus and midbrain. Previously, we have shown that loss of Nesp55 gives rise to alterations in novelty-related behavior. Here we extend these findings and demonstrate, using the *Nesp*$^{m/+}$ mouse model, that loss of Nesp55 leads to impulsive choices as measured by a delayed-reinforcement task, whereby *Nesp*$^{m/+}$ mice were less willing to wait for a delayed, larger reward, preferring instead to choose an immediate, smaller reward. These effects were highly specific as performance in another component of impulsive behavior, the ability to stop a response once started as assayed in the stop-signal reaction time task, was equivalent to controls. We also showed changes in the serotonin system, a key neurotransmitter pathway mediating impulsive behavior. First, we demonstrated that Nesp55 is co-localised with serotonin and then went on to show that in midbrain regions there were reductions in mRNA expression of the serotonin specific genes *Tph2* and *Slc6a4*, but not the dopamine specific gene *Th* in *Nesp*$^{m/+}$ mice; suggesting an altered serotonergic system could contribute, in part, to the changes in impulsive behavior. These data provide a novel mode of action for genomic imprinting in the brain and may have implications for pathological conditions characterized by maladaptive response control.

INTRODUCTION

Genomic imprinting is the process by which some genes are marked in a parent of origin specific manner as a consequence of epigenetic events that take place in the mammalian germ line (Ferguson-Smith, 2011). For canonical imprinted genes, in the somatic cell lineages this epigenetic marking leads to monoallelic expression from one parental allele only. For some imprinted genes, expression is solely from the maternal allele, whilst others are solely expressed from the paternal allele.

There are approximately 150 canonical imprinted genes and non-coding RNAs known in the mouse, with similar numbers in humans, although recent next-generation sequencing experiments have expanded this number (Wilkins *et al.*, 2016). Though relatively small in number, imprinted genes are critical for normal development to take place (Mcgrath & Solter, 1984, Surani *et al.*, 1984). Furthermore, functionally, imprinted genes converge on specific biological processes that have prominent importance in mammals, such as *in utero* growth, metabolism and behaviour (Peters, 2014, Wilkins *et al.*, 2016).

The imprinted gene *Nesp*, encoding Nesp55, is part of the complex *GNAS* imprinting cluster and is expressed exclusively from the maternal allele (Peters *et al.*, 1999). *Nesp* is expressed in discrete areas of the midbrain, including the Edinger-Westphal nucleus, dorsal Raphé nucleus (DRN), and the locus coeruleus (LC), and also in the hypothalamus (Plagge *et al.*, 2005). Our previous work demonstrated that mice null for maternally expressed Nesp55 show increased activity when placed in novel environment, but a reduced propensity to explore a novel environment when given the choice (Plagge *et al.*, 2005). Altered novelty exploration may involve changes in the balance between self-control and impulsive responding (Flagel *et al.*, 2010, Stoffel & Cunningham, 2008). Furthermore, *Nesp* is expressed in key brain structures, namely the DRN and LC, that are implicated in mediating response control (Bari & Robbins, 2013) and the ability to wait for a reward (Fonseca *et al.*, 2015), respectively. *A priori*, these data suggest possible links between Nesp55 and impulsive behavior.

The term 'impulsivity', defined as action with limited forethought, is a natural part of behavior. However, in certain individuals, impulsive behavior becomes

pathological and maladaptive and can manifest in psychiatric disease such as schizophrenia and attention deficit/hyperactivity disorder (ADHD), as well as addictive disorders such as pathological gambling and drug addiction. A growing body of evidence suggests that impulsivity is not unitary, and instead is a multi-faceted construct dissociable in terms of behavior and underlying neurobiology (Bari & Robbins, 2013). There have been attempts to segregate impulsive behavior into two separate categories, impulsive *choice* and impulsive *action*

(Broos *et al.*, 2012, Dent & Isles, 2014b). Impulsive choice often manifests as impulsive decisions resulting from a distorted evaluation of the delayed consequences of behavior and an increased preference for smaller immediate rewards over larger delayed rewards; on the other hand, impulsive action reflects the motoric aspect of impulsivity, and typically manifests in poor ability to override a pre-potent response (Humby & Wilkinson, 2011). A number of behavioral tasks exist to tease apart these discrete aspects of impulsivity (Humby & Wilkinson, 2011), many of which are readily available in mice (Dent & Isles, 2014b).

Here we used a delayed-reinforcement task (Isles *et al.*, 2003) and a stop-signal reaction time (SSRT) task (Humby *et al.*, 2013), to assay impulsive choice and impulsive action, respectively, in mice null for maternally expressed Nesp55 (*Nesp*$^{m/+}$ mice). We found that *Nesp*$^{m/+}$ mice showed a marked increase in choice impulsivity, manifest in decisions to choose an immediate, smaller reward over a larger but delayed reward, but exhibited no significant differences from control mice (*Nesp*$^{+/p}$) in impulsive action, where subjects had to suppress an ongoing pre-potent motor response. We also demonstrated that Nesp55 co-localizes with 5HT in midbrain regions, and further demonstrated that the behavioral effects observed in the *Nesp*$^{m/+}$ mice were accompanied by selective effects on the expression of key serotonergic genes. The data are consistent with a role for Nesp55 in mediating specific aspects of impulsive behavior, possibly via interactions with midbrain serotonergic pathways.

MATERIALS AND METHODS

Animals

All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986, under the remit of Home Office licence number 30/2673 with additional ethical approval at Cardiff University.

As described previously (Plagge *et al.*, 2005), expression of Nesp55 was eliminated by a deletion of 26 bp at the start codon of the ORF (bp 2362 to 2387 in AJ251761). Mice carrying a maternally derived null allele (*Nesp*$^{m/+}$) lacked Nesp55 expression, whereas those carrying a paternally derived null allele *Nesp*$^{+/p}$ have the same level of expression as wild-type mice (Figure S1). As before (Plagge *et al.*, 2005), in the present study *Nesp*$^{m/+}$ were compared to *Nesp*$^{+/p}$ mice as the most appropriate control (see Supporting Information for more details) and these are referred to as control mice throughout.

The Nesp55 null line was maintained on a C57Bl/6J (Charles River Laboratories, U.K.) background with separate cohorts of Nesp55 and control mice being utilized in the two behavioral tasks. Subjects were male mice aged between 4-10 months during testing; the large age range being due to the time taken to learn and perform the operant tasks. Standard laboratory chow was available *ad libitum*, but just prior to and during the experiment, water was restricted to 2h access per day. This regime maintained the subjects at ≈90% of free-feeding body weight. All behavioural testing was performed in the light phase (lights on 07:00, for 12-hours).

Behavioral tasks

Apparatus

The delayed-reinforcement task and stop-signal reaction time (SSRT) task were performed in 9-hole operant chambers (Cambridge Cognition Ltd, UK) modified for use in mice, as previously described (Humby *et al.*, 2013, Isles *et al.*, 2003). For the delayed-reinforcement task holes 3, 5 and 7 were open, whereas only holes 4 and 6 were open for the SSRT task. The mice were presented with visual stimuli (lights) recessed into the holes and were trained to respond to this

stimulus with a nose-poke recorded by infra-red beams spanning the hole. Reward was presented in a recessed compartment concealed by a panel, on the wall opposite to the nose-poke/stimulus array; animals were required to push the panel open in order to consume the reward. The control of stimuli and recording of the responses were managed by an Acorn Archimedes computer with additional interfacing by ARACHNID (Cambridge Cognition Ltd, UK).

Delayed reinforcement task

Details of the shaping procedures and basic aspects of the delayed-reinforcement task itself can be found elsewhere (Isles *et al.*, 2003). Briefly, the task comprised of three sequential blocks of 12 trials, with each trial consisting of an initial nose-poke to the centrally located stimulus, followed by a second nose-poke to either the left or right apertures. Trials 1-4 in any block were 'forced' information trials, where the initial nose-poke resulted in presentation of only one of the two choice options. This measure was designed to provide the subjects with prior notice of the extent of any delay associated with choosing the large reward. In the remaining 8 trials of each block, designated as 'choice' trials, the initial centre nose-poke led to the option of a second nose-poke response to either the left or right apertures. One response resulted in the delivery of a large reward (50µL 10% solution of condensed milk; Nestle Ltd, UK), and the other in the delivery of small reward (25µL 10% solution of condensed milk). The response contingencies were kept constant for each mouse, but were counterbalanced between subjects. In block 1 both responses led to the delivery of reward after a 1s delay. In blocks 2 and 3 increasing delays were introduced between the response and the delivery of the large reward (8s, 16s, respectively) whereas the delay between response and delivery of the small reward was fixed at 1s. As a probe to test the effect of the delays on behaviour, sessions were conducted where the delay associated with the large reward was fixed at 1s, equivalent to that associated with the small reward, throughout all three blocks of the session.

The bias in choice of the larger reward at each block (whereby always choosing the large reward=1; never choosing the large reward=0) was the main measure used to determine impulsive responding. Additional measurements that related to general motoric competence and motivation within the task were also

monitored, including the 'Start' and 'Choice' latencies, the time taken to initiate a trial and the time taken to make a choice once a trial was initiated, respectively. Also measured were the number of 'Non-started' (no initial, central nose-poke) and 'Omitted' (no secondary, choice nose-poke, following central nose-poke initiating trial) trials.

For the delayed reinforcement task experimental groups were *Nesp*$^{m/+}$=11, controls=7. Animals were tested 6-days per week.

Stop-signal reaction time (SSRT) task

Details of the shaping procedures and basic aspects of the main SSRT task can also be found elsewhere (Humby *et al.*, 2013). The SSRT task itself consisted of sessions of 100 trials, which involved both 'go' and 'stop' trials. Go trials consisted of rapid double nose-pokes (a 'go' response) between two separate stimuli locations, which were rewarded with reinforcement (22ul, 10% solution of condensed milk, Nestle Ltd, UK). 20% of trials were stop trials, pseudo-randomly distributed throughout each session, where a stop-signal (65db white noise for 0.3s) was presented between the first and second nose-poke responses. The aim of the stop-signal was to inhibit ('Stop') the mouse from making the second ('Go') nose-poke, and then wait for the reward. Failure to refrain from making this pre-potent response was punished by the absence of reward and 5 second time out (chamber light on). At baseline, the stop-signals were presented concurrently with the initial nose-poke response. To maintain high levels of performance of both go and stop responding, the go stimulus duration and wait period to reward delivery in a stop-signal trial were determined individually for each subject. To assess the ability to stop once an action had been initiated, sessions were implemented in which the onset of the stop-signal was presented at different positions within the individualised go response of each mouse. Thus, the stop-signal was pseudo-randomly presented 10, 40, 50, 60 and 90% from the onset of the go response of each subject, with the assumption that stopping would be more difficult the closer the stop-signal presentation was to the termination of the go response.

The amount of correct stopping in stop-signal trials and the SSRT were the main measures of impulsive responding in this task. The SSRT was calculated by

determining the 50% stopping ability for each subject from the range of sessions in which the stop-signal onset was varied from baseline (full details of this calculation can be found in the Supporting Information and (Humby *et al.*, 2013). The proportion of correct go-responses, and latency to respond, were also assessed. Additional measurements that related to general motoric competence and motivation within the task were also monitored, including the "intitiation" and "magazine" latencies, the time taken to initiate a trial and the time taken to collect the reward. Also measured was the number of trials completed for any given session.

For the SSRT task experimental groups were *Nesp*$^{m/+}$=17, controls=12. Animals were run 6-days per week.

Immunohistochemistry

Mice were terminally anaesthetised using an intraperitoneal injection of pentobarbitone and then trans-cardially perfused using 4%PFA in PBS. Brains were dissected whole and placed in 4% PFA overnight, then transferred to a 30% sucrose solution (in PBS) at 4˚c for 24 hours in order to dehydrate and cryoprotect them. Brains were then mounted on to a microtome platform using an embedding matrix and allowed to freeze fully. Serial sections of 40µm were sliced and put into 25-well containers containing cryoprotectant (6 sections per well). The free-floating sections in cryoprotectant were stored at -20°C until required.

Dual-labelling immunofluorescence of *Nesp* with 5HT was carried out in *Nesp*$^{+/+}$ (i.e. wild-type) free-floating brain sections in order to localize the endogenous protein. The Nesp55 primary antibody is a well-characterised anti-body (generated and obtained from the Reiner Fischer-Colbrie Lab). Specifically, it is a rabbit anti-NESP55 polycolonal anti-body, recognizing the free terminal end (GAIPIRRH) of NESP55 (Ischia *et al.*, 1997). Sections were washed three times for 10 min each in 0.1% PBS before being incubated for 15 min in 0.3 M glycine in 0.1% PBS at room temperature, to neutralise endogenous aldehyde groups. Sections were washed in 0.1% PBS and then incubated at room temperature for 1 hour in 10% blocking solution; 0.5% BSA (BB International, Cardiff, UK), 0.5%

Triton X-100 (v/v, Sigma Aldrich) in 0.1% PBS. Sections were then transferred to a solution containing 1:1000 anti-NESP55 and 1:500 anti-serotonin (Abcam) diluted in a 1% blocking solution; this was allowed to incubated overnight at 4°C whilst gently shaking. The next day sections were washed three times for 10 minutes in 0.1% PBS. The relevant fluorescent secondary antibodies (Alexa Fluor; Life technologies) were diluted 1/1000 in 1% blocking solution, sections were incubated in this solution in the dark at room temperature for 2 hours, whilst gently shaking. Sections were then washed in 0.1% PBS as before (in the dark) and transferred to polysine coated slides and allowed to dry over-night in a dark dust-free environment. The mounted slides were then dehydrated through a process of incubation in a rising concentration of alcohol, followed by xylene, then cover-slipped and sealed using DPX (Raymond Lamb DPX), and allowed to dry over-night. To control for non-specific binding of the secondary antibodies, secondary-only negative controls were carried out alongside all experiments.

Immunofluorescence slides were viewed and images captured using an upright fluorescence microscope (Leica DM5000 B). Dual-labelled immunofluorescence images were acquired through separate channels then subsequently merged using ImageJ (Image>colour>merge channels).

Quantitative PCR

RNA from macro-dissected brain regions was isolated using standard Trizol methods. Equal amounts of RNA were reverse transcribed using RNA to cDNA EcoDry (double primed) premix strips (Takara Bio Europe, France). Gene expression was assessed using a Rotorgene 6000 with a CAS1200 automated set up (Corbett Research, U.K.). PCR reactions were carried out using custom designed primers and Quantace SensiMix NoRef (Bioline). The genes assayed were *Th* (For 5′-AGGAGAGGGATGGAAATGCT-3′; Rev 5′-GCGCACAAAGTACTCCAGGT-3′)*, Tph2* (For 5′-CTGCTGTGCCAGAAGATCATCA-3′; Rev 5′-TGCTGCTCTCTGTGGTGTCG-3′) and *Slc6a4* (For 5′-TTGTGCTCATCGTGGTCATC-3′; Rev 5′-GTGGCGTACTCCTCCAGCAG-3′). Additionally, three housekeeping genes were assessed in order to provide a

robust measurement of general gene activity. These *Gapdh* (For 5′-GAACATCATCCCTGCATCCA-3′; Rev 5′-CCAGTGAGCTTCCCGTTCA-3′), *Hprt* (For 5′-TTGCTCGAGATGTCATGAAGGA-3′; Rev 5′-AATGTAATCCAGCAGGTCAGCAA-3′) and β-*actin* (For 5′-TCTGTGTGGATTGGTGGCTCTA-3′; Rev 5′-CTGCTTGCTGATCCACATCTG-3′). ΔCt values were generated by normalising to the geometric mean of these three housekeeping genes. All individual reactions were carried out in triplicate. Real time qPCR data was visualised using the ΔΔCt method (Livak & Schmittgen, 2001).

Data analysis and statistics

All behavioral data were analysed using SPSS 20 (SPSS, USA). Data were assessed for normality and then analysed by Student's t-test or mixed ANOVA, with between-subjects factors of GENOTYPE (*Nesp*$^{m/+}$ vs. Control), and within-subject factors DELAY (1s,8s,16s, or 1s,1s,1s), CHOICE (choice of large or small reward during forced trials of the delayed reinforcement task), and STOP-SIGNAL POSITION (position of stop-signal relative to individualized Go-response). For repeated-measures analyses, Mauchly's test of sphericity of the covariance matrix was applied; significant violations from the assumption of sphericity were subject to the Huynh–Feldt correction to allow more conservative comparisons through adjust degrees of freedom. Due to the non-parametric nature of the qPCR data, ΔCt values were analysed by Mann-Whitney U test. All significance tests were performed at alpha level of 0.05.

RESULTS

Nesp$^{m/+}$ mice are more impulsive in the delayed reinforcement task

In the delayed-reinforcement task, subjects made a choice between receiving a small food reward after a short (1s) delay, or a large reward after a longer delay (1, 8 or 16s). In this way, the extent to which subjects made impulsive choices, indexed by the preference for choosing the immediate but small reward over a delayed large reward, was determined. Increasing the delay to the large reward within a session decreased the likelihood of choosing that reward for both *Nesp*$^{m/+}$ and control mice (Figure 1a, main effect of DELAY, $F_{2,32}$=3.84, P=0.032).

However, mutant and control mice showed significant differences in the extent to which they switched their preference to the small, less delayed reward in response to increased delay to the large reward. Specifically, *Nesp*$^{m/+}$ mice chose the small reward more readily than control mice (Figure 1a, main effect of GENOTYPE, $F_{1,16}$=6.74, P=0.019), indicative of increased impulsive responding. To confirm the specificity of the contingency between reward value and the delay in reward delivery, performance was also assessed where the cost associated with the large reward was removed, thus the delay associated with the large and small rewards was equal (1s) for all trials. In these sessions, all subjects demonstrated an equal high preference for the large reward throughout the session (Figure 1b, no main effect of DELAY, $F_{2,32}$=0.478, P=0.624) and there were no differences between *Nesp*$^{m/+}$ and control mice (no main effect of GENOTYPE, $F_{1,16}$=0.010, P=0.927).

We also confirmed that the contrasting pattern of choice behavior in the *Nesp*$^{m/+}$ mice were not due to any differences between the groups in terms of basic learning and motivation to carry out the task, as *Nesp*$^{m/+}$ animals acquired the task at the same rate as control mice (number of sessions to last day of baseline, grand mean= 40.3 ±1.3 SEM, t_{16}=1.00, P=0.32). Additionally, variability between *Nesp*$^{m/+}$ and control mice was not related to differences in experiencing the information trial contingencies. Thus, in the 'forced' trials (in which no choice was available) both *Nesp*$^{m/+}$ and control mice made equal responses to the large and small reward-related stimuli at all delays (interaction between GENOTYPE x DELAY x CHOICE, $F_{2,32}$=1.85, P=0.43; data not shown). Finally, there was no difference between *Nesp*$^{m/+}$ and control mice on general measures of task performance (see Figure S2). As expected, *Nesp*$^{m/+}$ and control mice demonstrated a general main effect of DELAY on many of these measures (Figure S2a-d) as follows: increased start ($F_{1.7,27.5}$=22.06, P<0.001) and choice latencies ($F_{2,32}$=4.01, P=0.028) with increasing delay; increased number of non-started trials ($F_{2,32}$=127.29, P<0.001) and omitted trials ($F_{1.7,26.4}$=5.48, P=0.014) with increasing delay. For all of these measures, there were no GENOTYPE differences or interactions between GENOTYPE and DELAY (see Table S1 for details of statistics).

Nesp$^{m/+}$ mice show no differences in impulsive responding in the stop-signal reaction time task

The SSRT task measures the ability to stop an action once initiated by presenting a stop-signal (in this case an auditory tone) during a rapid transition between two stimuli locations (the 'go' response). Correctly inhibiting the go response earned reward in a 'stop' trial, and the difficulty of stopping was manipulated by presenting the stop-signal at different times within the go response. Thus stopping was easiest when the stop-signal onset was near the start and more difficult when at the end of the go response. Throughout the training stages of the SSRT task, all subjects showed equivalent behavior in learning the task. *Nesp*$^{m/+}$ mice acquired the task at the same rate as control animals, as demonstrated by the similar number of sessions taken to complete the task (*Nesp*$^{m/+}$: 44.3±6.6, control: 46.6±6.8). The effects of altering the position of the auditory stop-signal during stop trials on stopping efficiency are illustrated in Figure 2a. In line with previous SSRT task experiments in humans and a variety of other mammalian species, including mice (Bari *et al.*, 2009, Humby *et al.*, 2013), stopping became increasingly difficult as the stop-signal presentation was moved progressively closer to the execution of the response (10, 40, 50, 60 and 90% into the individualised go response) leading to systematic reductions in the ability to stop for both *Nesp*$^{m/+}$ and control mice (Figure 2a, main effect of STOP-SIGNAL POSITION, $F_{4,108}$=43.28, P<0.001). However, there were no differences in stopping efficiency between *Nesp*$^{m/+}$ and controls (Figure 2a, main effect of GENOTYPE, $F_{1,27}$=0.58, P=0.45). The lack of differences in impulsive behavior in this task was further demonstrated by another measure of response inhibition, the speed of stopping or stop-signal reaction time (SSRT) derived, as is conventional (Bari *et al.*, 2009, Davies *et al.*, 2014, Humby *et al.*, 2013), in sessions where the subjects exhibited 50% correct stopping. Equivalent SSRTs were observed in both *Nesp*$^{m/+}$ and control mice (Figure 2b; t_{27}=17, P=0.87). There were also no differences in the Go response between the *Nesp*$^{m/+}$ and control mice, in terms of the amount (Figure 2c, main effect of GENOTYPE, $F_{1,27}$=0.16, P=0.69) or speed (Fig. 2d, main effect of GENOTYPE, $F_{1,27}$=0.20, P=0.66), of correct responding in go trials. Consistent with previous results (Humby *et al.*, 2013), these parameters were not affected in sessions when the

stop-signal position was moved from baseline (main effect of STOP-SIGNAL POSITION, $F_{4,108}$=0.49, P=0.74 and $F_{4,108}$=1.67, P=0.16, for the amount and speed of correct responding in go trials, respectively).

Again, *Nesp*$^{m/+}$ animals acquired the task at the same rate as control mice (number of sessions, grand mean=45.3 ±1.2 SEM, t_{27}=0.90, P=0.38). Additionally, there was no difference between *Nesp*$^{m/+}$ and control mice on general measures of task performance during individualized SSRT session). Thus, the number of trials completed (t_{27}=0.10, P=0.92), latency to initiate a trial (t_{27}=-0.36, P=0.73) and magazine latency (t_{27}=-1.12, P=0.27) measures were not significantly different between *Nesp*$^{m/+}$ and control mice, but indicated a high degree of stimulus control for both groups in the task (see Figure S3).

Nesp55 co-localises with 5-HT; key serotonin genes show reduced expression in mid-brain of Nesp$^{m/+}$ mice

Previous work (Bauer *et al.*, 1999, Plagge *et al.*, 2005), which is replicated here (Figure S1 d-f), shows that Nesp55 is strongly expressed in the hypothalamus and areas of the midbrain, including the Edinger-Westphal nucleus, DRN, and the LC. Using immunohistochemistry, we examined whether Nesp55 co-localises with 5-hydroxytryptamine (5HT) in these regions. Qualitative observation suggests that Nesp55 is indeed co-localised with 5HT in the midbrain (Figure 3 a-c). There appears to be very little, or no co-localisation of Nesp55 and 5HT in the hypothalamus (Figure 3 d-f). Additionally, it is clear that Nesp55 is also expressed non-5HT expressing cell-types.

To investigate the relationship between Nesp55 and 5HT, the expression levels of some 5HT-related genes were assessed by qPCR in samples of the midbrain of *Nesp*$^{m/+}$ and control mice (Figure 4). This brain region was chosen as it was the area showing the greatest co-localisation between Nesp55 and 5HT. There were 10-fold reductions in the expression of genes encoding tryptophan hydroxylase, *Tph2* (U=0.01, P=0.021) and the serotonin transporter, *Slc6a4* (U=0.01, P=0.021) in *Nesp*$^{m/+}$ mice relative to control subjects, but no difference in the expression of *Th* (U=7.0, P=0.886), the gene encoding tyrosine hydroxylase.

DISCUSSION

The imprinted gene *Nesp*, expressed from the maternal allele only, encodes Nesp55, and is located in discrete areas of the midbrain and hypothalamus. Here we demonstrate that loss of Nesp55 expression from the maternal allele, but not paternal allele, leads to higher levels of impulsive choice behavior as measured by a delayed reinforcement task. The *Nesp*$^{m/+}$ mice were less willing to wait for a larger but delayed reward, choosing instead the smaller but immediately available reward. These effects were highly specific in that behavior in the SSRT task, assaying a different form of behavioral control, the ability to stop the execution of a pre-potent action, was equivalent between *Nesp*$^{m/+}$ and control mice. In addition, we show that Nesp55 is co-localised with 5HT and that there are concomitant reductions in midbrain *Tph2* and *Slc6a4*, but not *Th* mRNA expression in *Nesp*$^{m/+}$ mice, suggesting an altered serotonergic system could contribute, in part, to the changes in impulsive choice behavior.

We have previously shown that *Nesp*$^{m/+}$ mice have an increased reactivity to novelty, but a reduced propensity to explore a novel environment when given a choice (Plagge *et al.*, 2005). Altered novelty exploration may involve changes in the balance between self-control and impulsive responding (Flagel *et al.*, 2010, Stoffel & Cunningham, 2008). We decided to explore the impulsivity phenotype of *Nesp*$^{m/+}$ mice further by using operant tasks to examine discrete aspects of impulse control, namely choice impulsivity, as measured on a delayed reinforcement task, and impulsive action, as measured on the SSRT task (Dent & Isles, 2014b). In the delayed reinforcement task, *Nesp*$^{m/+}$ mice showed higher levels of impulsivity, switching their choice of the larger, but increasingly delayed, reward, more quickly than control mice. Critically though, when the delay associated with the larger reward was equivalent to that associated with the smaller reward throughout the session, there was no difference in performance between *Nesp*$^{m/+}$ and control mice. Taken together with other control measures within the delayed-reinforcement task, which indicated that there were no differences between *Nesp*$^{m/+}$ and control mice in their ability to learn the task or their general motivation to perform the task, this manipulation demonstrates that the prime factor controlling the difference in behavior

between *Nesp*$^{m/+}$ and control mice on the delayed-reinforcement task, was the contingency between delay and reward. Conversely, behavior of *Nesp*$^{m/+}$ and control mice on the SSRT task, was equivalent. All mice showed the expected reduction stopping as the stop-signal presentation moved progressively closer to the execution of the go response, but there were no genotype-related differences across the task or in the derived SSRT measure.

Overall, these data suggest that loss of maternal Nesp55 expression produces a specific increase in impulsive choice behavior, whilst impulsive action remains unaffected. As a concept, the dissociation of choice and action impulsivity is now well established. For instance, inbred strains of mice have been shown to exhibit dissociations in terms of impulsive behaviors in a delayed reinforcement task (Isles *et al.*, 2004), and a measure of action impulsivity in the 5-choice serial reaction time task (Patel *et al.*, 2006). Furthermore, a cross-species study examining behavior in a number of different tasks found that, in both rats and humans, measures of impulsive choice and impulsive action did not correlate (Broos *et al.*, 2012). Our data add to the growing body of evidence suggesting that impulsivity is not unitary, and instead is a multi-faceted construct.

Impulsive choice and impulsive action are not only dissociable behaviorally, but also in terms of the underlying neurobiology (Bari & Robbins, 2013). One neural pathway that has important dissociable effects on discrete aspects of impulsive behavior is the serotonin system (Bari *et al.*, 2009, Humby *et al.*, 2013, Talpos *et al.*, 2006, Winstanley *et al.*, 2004). Although excluded from the key areas of cognitive control important for mediating impulsive behavior, such as the frontostriatal circuitry, Nesp55 is strongly expressed in a number of serotonergic areas of the midbrain (Bauer *et al.*, 1999, Plagge *et al.*, 2004), and here we show that in some cells Nesp55 and 5HT are co-localised. This is particularly interesting given the recently established role of serotonergic dorsal raphé neurons in mediating waiting for delayed reward (Miyazaki *et al.*, 2012a, Miyazaki *et al.*, 2012b), independent from any effects on reward (Fonseca *et al.*, 2015). One mechanism underlying the specificity of impulsive behavior phenotype seen in *Nesp*$^{m/+}$ mice could be via an imbalance in serotonin signaling in the DRN.

Quantitative PCR analysis revealed that in mice lacking Nesp55 there was a 10-fold reduction in midbrain expression of *Tph2*, which encodes tryptophan hydroxylase the rate limiting enzyme for brain 5HT synthesis (Fitzpatrick, 1999), and the serotonin transporter gene, *Slc6a4*. Expression of *Th* (tyrosine hydroxylase) was unaltered, consistent with a specific effect on the serotonin system. Nesp55 is associated with fast anterograde axonal transport in the peripheral nervous system and is considered a marker for the constitutive secretory pathway (Fischer-Colbrie *et al.*, 2002, Li *et al.*, 2002). It is unknown whether Nesp55 influences the transport or release of neurotransmitter vesicles. We have previously found no alteration in monoamine levels in brain tissue taken from the prefrontal cortex and midbrain of *Nesp*$^{m/+}$ mice (Plagge *et al.*, 2005). However, these whole tissue analyses may mask the impact of Nesp55 deficiency on neurobiological indices more closely related to synaptic function, such as extracellular levels of transmitters and changes in pre- and postsynaptic receptor moieties, an idea maintained by the correlative changes in *Slc6a4* expression. Moreover, given the known interplay between the serotonin and dopamine systems in influencing impulsive behaviour (Dalley & Roiser, 2012), it is not possible to rule out the action of other monoamines in *Nesp*$^{m/+}$ mice as being causal of the delayed-reinforcement phenotype. Therefore, at present the exact neural mechanism(s) by which Nesp55 influences behavior remains unclear.

Our data provide a novel mode of action for genomic imprinting in the brain, adding to the list of behaviors sensitive to imprinted gene function (Davies *et al.*, 2015). Imprinted genes are thought to have evolved as a consequence of conflicting phenotypic "interests" between maternal and paternal genes, which causes an escalating arms race in relation to allelic expression, eventually leading to silencing of one or other parental allele (Moore & Haig, 1991, Wilkins & Haig, 2003). In addition to the allelic level, parental conflict may also be manifest at the gene and tissue levels (Davies *et al.*, 2005, Wilkins *et al.*, 2016). Evidence of this parental conflict is seen in the opposite action of imprinted genes during *in utero* growth and early post-natal life (Haig, 2004) but as yet there are no behavioural examples. Our findings may add some support to the theory that, as a consequence of intra-genomic conflict, imprinted genes expressed in the adult

brain could lead to a "parliament of the mind" with regard to decision-making (Haig, 1997) in that there are opposing parental interests pulling impulsive choice in different directions. However, whilst here we show that loss of a maternally expressed gene, *Nesp*, causes an increase in impulsive choices, there is, currently at least, no paternally expressed imprinted gene that has been shown to influence impulsive responding. A potential candidate here is *Grb10*, which shows overlapping expression with *Nesp* (Dent & Isles, 2014a), and influences social dominance (Garfield *et al.*, 2011), a behavioral domain that has been linked to impulsivity (Davis *et al.*, 2009). Finally, an extension of this idea and our experimental findings is that, an imbalance in expression of imprinted genes, such as *Nesp*, may not only contribute to facets of impulsivity within the normal range, but also to pathological conditions, such as gambling and drug addiction, where response control becomes maladaptive. It is therefore, of particular interest that a *NESP* deletion polymorphism has been associated with the occurrence of neuropsychiatric illness, including addictive behaviour (Kim *et al.*, 2000).

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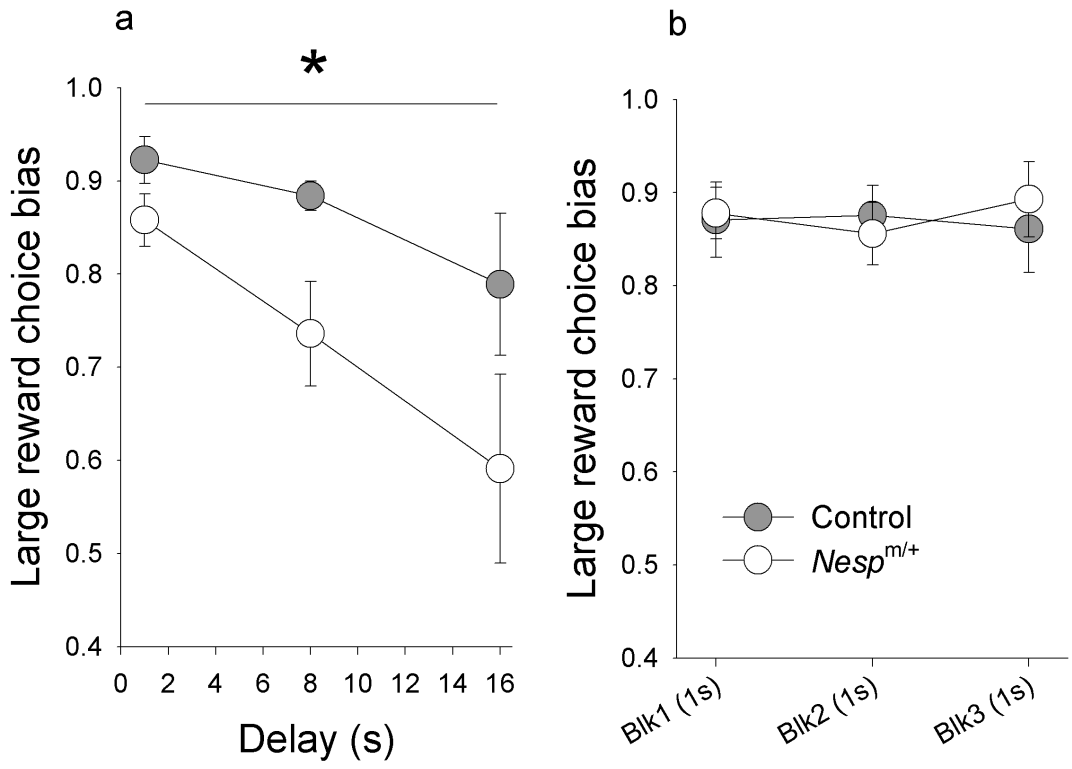

Figure 1. *Nesp*$^{m-/p+}$ mice show increased impulsive choice behavior in the delayed-reinforcement task. Behavior of both *Nesp*$^{m/+}$ and control mice changed across session blocks (blk) with increasing delay, such that choice bias moved away from the response leading to the large reward towards the small reward with increasing delay (**a**). However, there were systematic differences between the groups in their behavior, such that *Nesp*$^{m/+}$ animals (N=11) switched their choice to the small, less delayed reward more quickly than control mice (N=7). When the delay associated with the large and small rewards was equal (1s) throughout the session (b), choice bias was consistently high (large reward chosen approximately 90% of the time). Moreover, under these conditions there were no differences in choice bias between *Nesp*$^{m/+}$ and control mice. Data shows mean ±SEM of three consecutive stable sessions; * represents $P<0.05$ main effect of DELAY; # represents main effect of GENOTYPE.

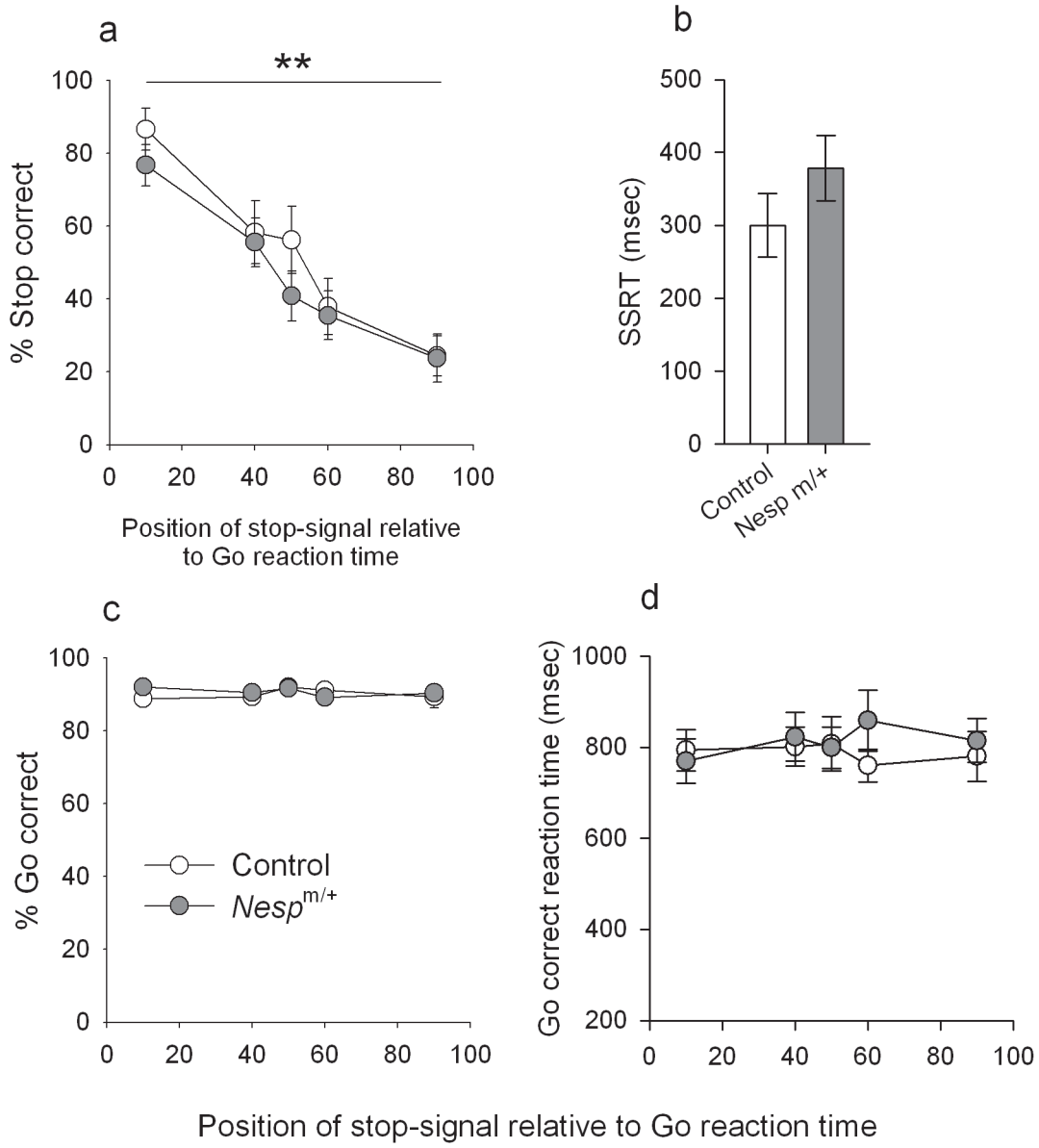

Figure 2. No difference between *Nesp*$^{m/+}$ and control mice performance in the stop-signal (SSRT) task. Both control and *Nesp*$^{m/+}$ mice showed an equivalent ability to perform the SSRT task showing the expected change in percentage correct responding during a 'stop' trial (**a**) as the position of the stop-signal was altered, but there were no differences between *Nesp*$^{m/+}$ (N=17) and control mice (N=12). *Nesp*$^{m/+}$ and control mice also showed equivalent SSRTs at 50% correct stopping (**b**). There were no genotype differences for the 'Go' response for both cohorts of mice, in terms of percentage correct responding **(c)** or response speed (**d**). Data shows mean ±SEM; ** represents P<0.01 main effect of stop-signal position.

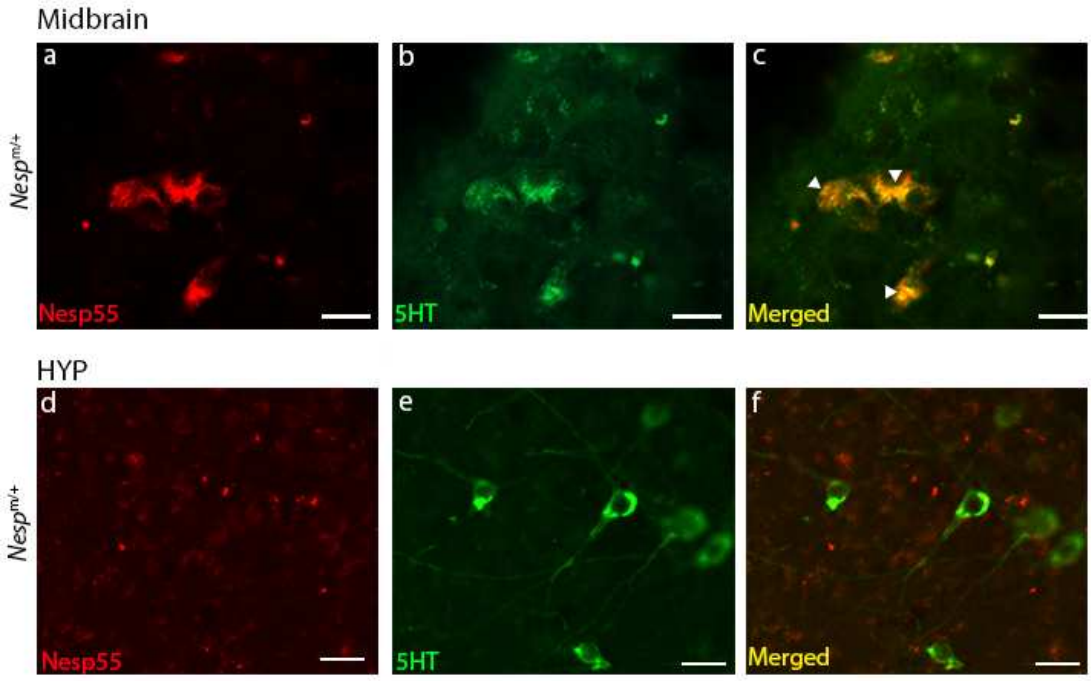

Figure 3. Co-localisation of Nesp55 with 5HT in the brain. Sections were dual-labelled with anti-bodies against Nesp55 and 5HT, images were then merged to gauge cellular co-localisation. White arrows depict areas of co-localisation. There was evidence of co-localisation in the regions of the midbrain (a-c), for example the locus coeruleus. However, there was very little co-localisation observed in the hypothalamus (d-e). All images are at x40 magnification

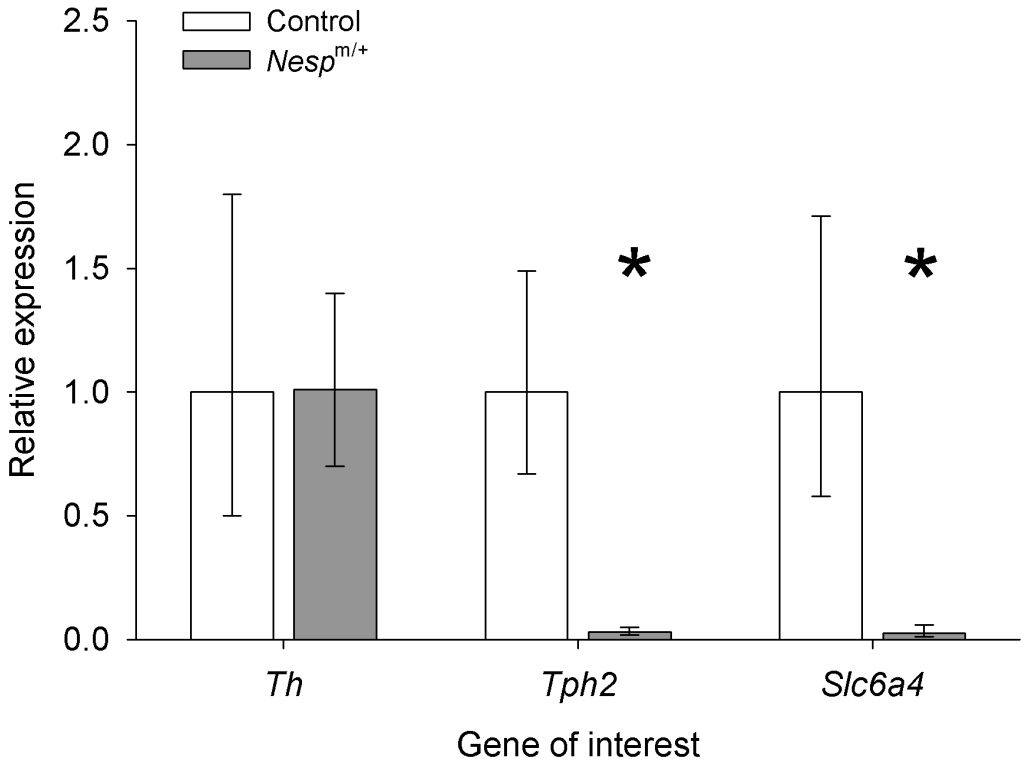

Figure 4. Altered 5HT-related gene expression in *Nesp*$^{m/+}$ mice. Gene expression analysis of midbrain macro-dissections from *Nesp*$^{m/+}$ and control mice (N=4 for both groups) showed significant 10-fold reductions in *Tph2* and *Slc6a4* expression, but no difference in *Th* expression. Data shows mean ±SEM; * represents P<0.05.

Impulsive choices in mice lacking imprinted Nesp55 - supplementary material

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SUPPLEMENTARY MATERIALS AND METHODS

Animals

Loci closely linked to a targeted mutation may cause concern with regard to phenotypic differences between knock-outs and controls, since they most likely retain the original 129 inbred strain ES cell alleles, even after several generations of backcrossing to a different strain. For analysis of mice carrying a homozygous mutation of a biallelically expressed gene, this may imply comparing an associated homozygous 129/Sv genetic background with wild-type C57BL/6J counterparts. However, the knockout analysis of monoallelically expressed genes provides the opportunity for a more direct comparison of heterozygous groups. In the case of maternally expressed *Nesp*, heterozygous deficient mice (*Nesp*$^{m/+}$)can be compared with heterozygous Nesp-expressing mice (*Nesp*$^{+/p}$) obtained from reciprocal crosses to C57BL/6J. In both of these groups, the loci

surrounding the targeted mutation will comprise compound C57BL/6J and 129/Sv alleles, and thus, any potential strain variations should manifest equally in both groups. In contrast, the wild-type littermates of the two heterozygous groups are homozygous C57BL/6J for these genomic regions, could confound the interpretation of behavioral assays (Gerlai, 1996), particularly given known differences in impulsive behavior between inbred strains (Isles *et al.*, 2004). Thus, we considered it most appropriate to compare *Nesp*$^{m/+}$ with *Nesp*$^{+/p}$ mice as controls.

Immunohistochemistry

For the Immunohistochemistry analysis a Vectastain® Elite ABC Kit (PK-6100) was used on brain sections from *Nesp*$^{m/+}$ (lacking maternal Nesp55), *Nesp*$^{+/P}$ (control, carrying paternal knockout), and wild-type (*Nesp*$^{+/+}$) mice. Sections were first washed in TBS (4 x 10mins). They were then incubated in a peroxidase block on a stirrer for 30 minutes (0.6% hydrogen Peroxidase in TBS) to block endogenous peroxidase. Sections were washed in TBS again (3 x 10min in TBS), and then incubated in TBST with 3% normal goat serum (NGS) (S-1000, vector labs) on a stirrer for 30 minutes at room temperature. Following NGS blocking, sections were incubated in primary antibody (anti-Nesp55) diluted at 1:1000 in TBST with 3% NGS. This was stirred for 10 minutes and then covered and stored overnight at 4˚c. The following day the sections were washed 3x 10 minutes in TBST with 3% NGS. Sections were then incubated for 1 hour in the secondary antibody diluted 1:200 in TBST with 3% NGS at room temperature. Following the secondary antibody incubation sections were washed 3 x 10 minutes in TBST, and then allowed to incubate in the ABC complex (made as per kit specifications) at room temperature, on a stirrer for 1 hour. Sections were then washed as before (3x 10 minutes in TBST), then washed 2 x 10 minutes in 0.05M Tris buffer. Sections were then incubated in DAB solution for 35 seconds at room temperature; they were then immediately washed in cold PBS in order to stop the reaction. Finally sections were then washed in TBST for 2 minutes and left in a new change of TBST solution overnight. The following day sections were mounted on to polysine coated slides, and allowed to dry over-night. The mounted slides were then dehydrated through a process of incubation in a rising

concentration of alcohol followed by xylene, then cover-slipped and sealed using DPX (Raymond Lamb DPX), and allowed to dry over-night. All experiments were carried out alongside negative controls.

Calculating the Stop Signal Reaction Time

Correct go reaction times were determined directly, however the stop-signal reaction time (SSRT) had to be derived from the distribution of correct go reaction times and the proportion of correctly stopped trials. SSRTs in the task were estimated employing the standard procedure described in Logan et al. (1984), using data from where the proportion of correct stop responses was ~50%. For each subject, data from the sessions in which the stop-signal positions were varied relative to the individualised go reaction time, were ranked by the proportion of correct stop responses, and data from sessions in which this value was between 40% and 60% (i.e. 50%±10%) were averaged. The latency of stopping as defined by the SSRT was derived from the distribution of correct go reaction times and the proportion of correctly stopped trials as previously described (Eagle & Robbins, 2003, Logan, 1994). Hence, for each of the sessions determined above, the correct go reaction times were rank ordered from smallest to largest and the n^{th} value found, where n is the rank order position based on the proportion of failing to stop correctly in stop trials was corrected for the occurrence of omitted go trials. Although omitted go trials were a rare occurrence, this correction was implemented based on the rationale that they could alter the observed inhibition function and affect determination of the n^{th} correct go reaction time value and hence the final SSRT (Eagle & Robbins, 2003, Solanto *et al.*, 2001, Tannock *et al.*, 1989). To determine the SSRT, the time the stop-signal was presented (i.e. 'mean correct go reaction time' x '% mean stop-signal position') was subtracted from the n^{th} correct go reaction time value.

SUPPLEMENTARY RESULTS

Initial validation of the Nesp55 anti-body was carried out by completing immunohistochemistry staining on brain sections from WT, *Nesp*$^{m/+}$ and *Nesp*$^{+/p}$ adult mice (Figure S1). The staining revealed that the *Nesp* anti-body successfully targets the Nesp55 protein, showing cell specific staining in both WT and *Nesp*$^{+/p}$ (whereby maternal Nesp is present in the brain); in the hypothalamus, pons and mid-brain regions, consistent with the discrete regions previously described

(Plagge *et al.*, 2005); and showing no staining in the *Nesp*$^{m/+}$ sections (whereby maternal Nesp55 has been deleted).

Additional measures in the delayed reinforcement task

In addition to choice, a number of other measures of basic behavior within the delayed reinforcement task were obtained. These relate to general motoric competence and motivation within the task. As expected, *Nesp*$^{m/+}$ and control mice demonstrated a general main effect of DELAY on many of these measures (Figure S2) as follows: start ($F_{1.7,27.5}$=22.06, P<0.001) and choice latencies ($F_{2,32}$=4.01, P=0.028); and non-started trials ($F_{2,32}$=127.29, P<0.001) and omitted trials ($F_{1.7,26.4}$=5.48, P=0.014). In all of these measures *Nesp*$^{m/+}$ and control mice showed a high degree of stimulus control, with no GENOTYPE differences or interactions between GENOTYPE and DELAY (see Table S1).

Measure	Main effect of GENOTYPE		Interaction: GENOTYPE and DELAY	
	F	P	F	P
Start latency	2.14	0.16	0.70	0.48
Choice latency	0.05	0.82	0.24	0.69
Non-started trials	1.69	0.21	0.46	0.64
Omitted trials	0.01	0.99	0.07	0.90

Table S1 Statistical values for additional measures of performance of *Nesp*$^{m/+}$ and *Nesp*$^{+/p}$ mice within the delayed-reinforcement task. Analysis of main effect of GENOTYPE, and interaction between GENOTYPE and DELAY.

Additional measures in the SSRT task

In addition to the main measures, a number of other measures of basic behavior within the SSRT task were obtained (Figure S3). Details of these measures and their statistical analyses, can be found in the main text

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Figure S1

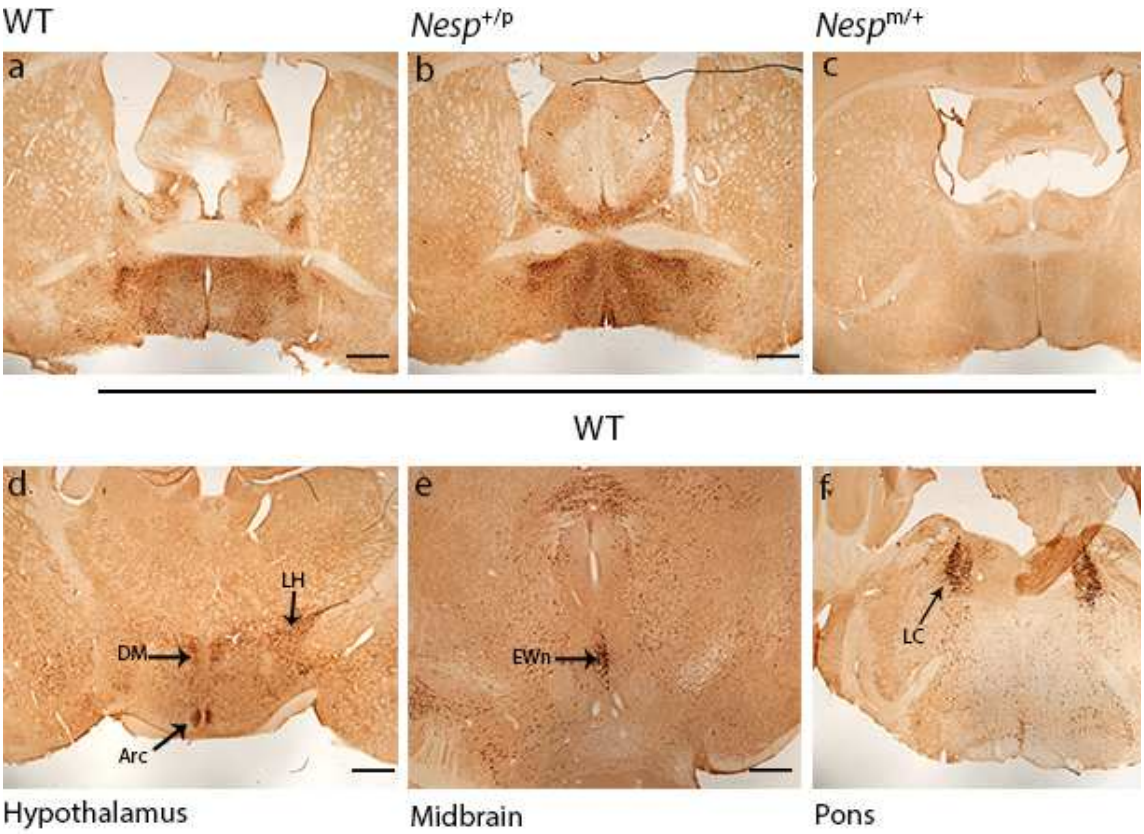

Figure S1 Immunohistochemistry staining for Nesp in coronal sections of the adult mouse brain. (a-c) Shows staining in WT, *Nesp*$^{+/p}$ (used as controls) and *Nesp*$^{m/+}$ adult mice; showing the presence of discrete expression of Nesp55 in the hypothalamus in both WT (a) and *Nesp*$^{+/p}$ (b) mice, but not *Nesp*$^{m/+}$ mice (c). (d-f) WT mouse brain probed using anti-Nesp55 antibody shows distinct staining in the hypothalamus, specifically the DM, dorsomedial hypothalamic nucleus, Arc, arcuate hypothalamic nucleus, and the LH, lateral hypothalamic area (d); in the midbrain, specifically the EWn, Edinger-Westphal nucleus (e); and in the pons, specifically the LC, Locus Coeruleus (f).

Figure S2

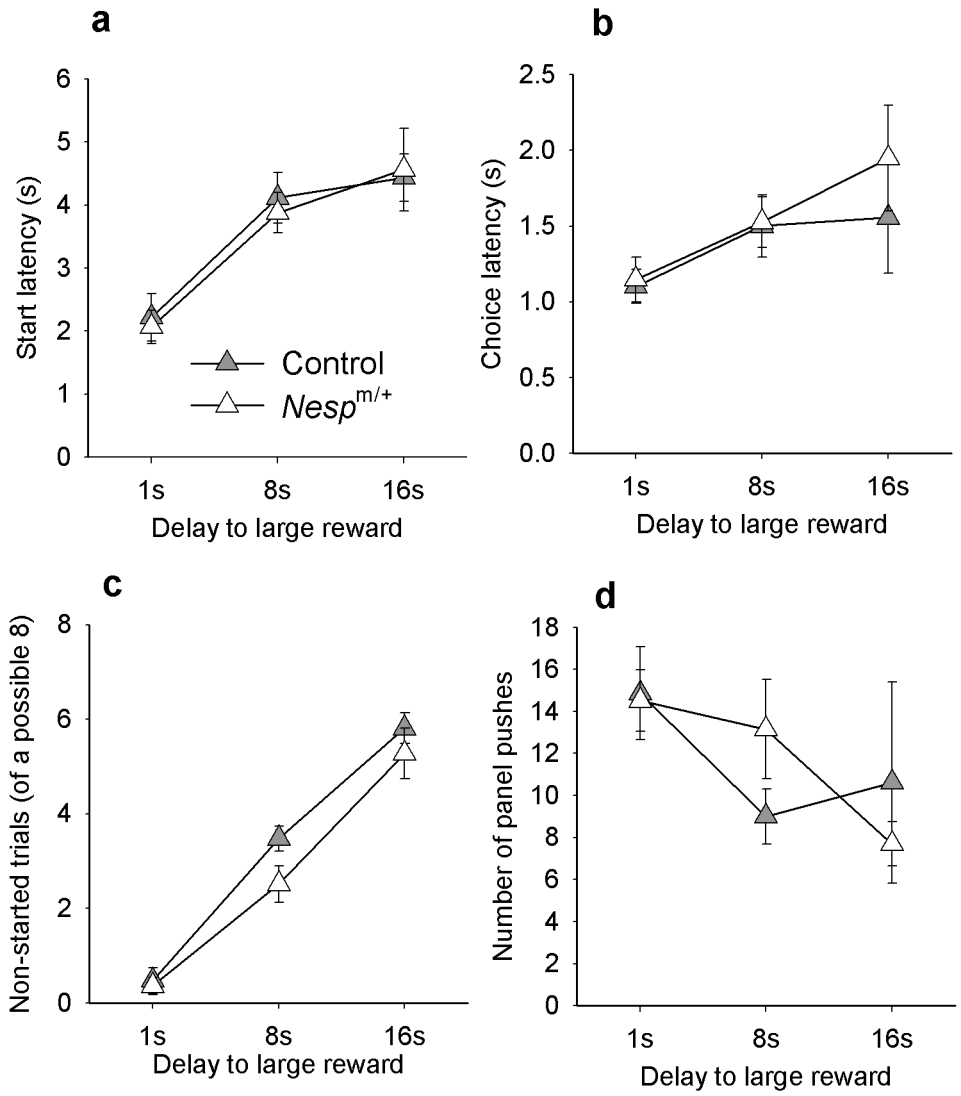

Figure S2 Additional behavioral measures of performance of *Nesp*$^{m/+}$ and control mice in the delayed reinforcement task. a Start latency; **b** Choice latency; **c** Non-started trials; **d** Panel pushes. All data represent means ±s.e.m.

Figure S3

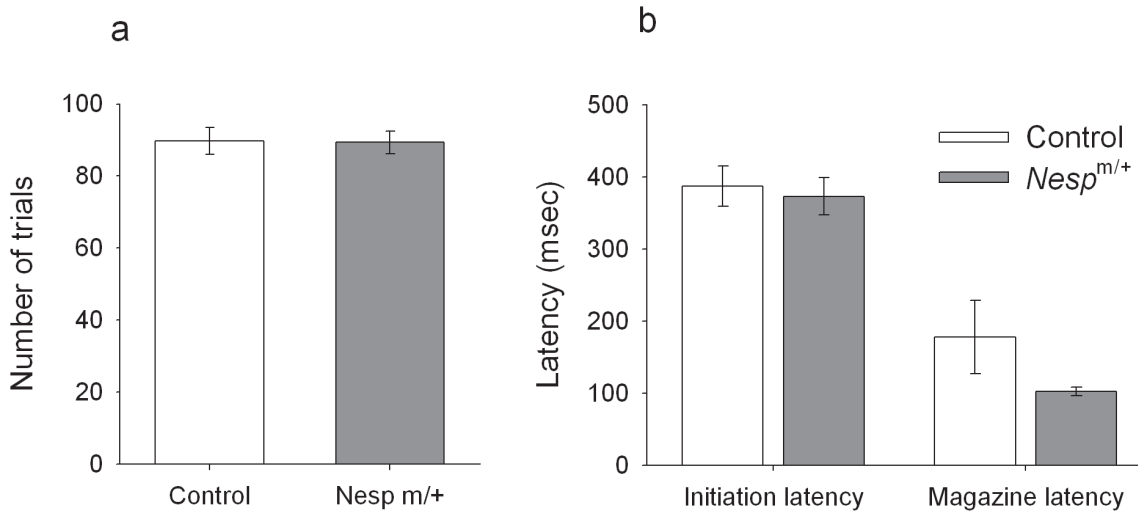

Figure S3 Additional behavioral measures of performance of *Nesp*$^{m/+}$ and control mice in the SSRT task. a Number of trials in a session with individualised SSRT (when the stop cue is presented 50% into the individual's Go-reaction time); **b** Initiation and magazine latency in a session with individualised SSRT. All data represent means ±s.e.m.