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Histone deacetylase inhibitor treatment promotes spontaneous caregiving behaviour in non-aggressive virgin male mice

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Abstract

The majority of mammalian species are uniparental, with the mother solely providing care for young conspecifics, although fathering behaviours can emerge under certain circumstances. For example, a great deal of individual variation in response to young pups has been reported in multiple inbred strains of laboratory male mice. Furthermore, sexual experience and subsequent cohabitation with a female conspecific can induce caregiving responses in otherwise indifferent, fearful or aggressive males. Thus, a highly conserved parental neural circuit is likely present in both sexes; however, the extent to which infants are capable of activating this circuit may vary. In support of this idea, fearful or indifferent responses toward pups in female mice are linked to greater immediate early gene (IEG) expression in a fear/defensive circuit involving the anterior hypothalamus compared to that in an approach/attraction circuit involving the ventral tegmental area. However, experience with infants, particularly in combination with histone deacetylase inhibitor (HDACi) treatment, can reverse this pattern of pup-induced activation of fear/defence circuitry and promote approach behaviour. Thus, HDACi treatment may increase the transcription of primed/poised genes that play a role in the activation and selection of a maternal approach circuit in response to pup stimuli. In the present study, we investigated whether HDACi treatment would impact behavioural response selection and associated IEG expression changes in virgin male mice that are capable of ignoring, attacking or caring for pups. The results obtained indicate that systemic HDACi treatment induces spontaneous caregiving behaviour in non-aggressive male mice and alters the pattern of pup-induced IEG expression across a fear/defensive neural circuit.

KEYWORDS
histone acetylation, immediate early gene expression, Npas4, paternal behaviour

1 | INTRODUCTION

Mus musculus is a uniparental rodent species in which the mother solely cares for her young in the wild. However, maternal-like behaviour (pup retrieval, sniffing/licking, crouching) along with the elimination of pup-directed aggression has been reported in commonly used laboratory strains of male mice.[1-5] These caregiving behaviors have been observed in both sexually experienced males, which are often cohabited with females to optimally produce offspring, as well as sexually naïve male mice, albeit much less frequently. When exposed to pups, sexually naïve male mice tend to show highly variable responses to pups, including aggressive, exploratory, avoidant, and even spontaneous caregiving

behaviours.[6] Taken together, these data support the idea that the neural circuit that regulates maternal behaviour is conserved in male mice, although the extent to which infants activate this circuit varies considerably between individuals as a result of largely unknown mechanisms.

In females, seminal work uncovering the neural mechanisms that gate infant stimulation of the maternal neural circuit was conducted in postpartum rats[7] and recent work has replicated some of these findings in mice.[8,9] Importantly, motivation to care for offspring first occurs around the time of birth. In non-parental animals, infants activate hypothalamic regions known to regulate anxiety/escape/attack behaviours such as the anterior hypothalamic nucleus (AHN) and ventromedial nucleus of the hypothalamus (VMN).[6,10,11] Furthermore, lesions of these hypothalamic attack regions promote the onset of maternal behaviour in sub-optimally hormonally primed nulliparous female rats.[10,12] By contrast, the medial preoptic area (mPOA) of the rostral hypothalamus regulates caregiving behaviour via its projection to the ventral tegmental area (VTA), which drives the release of dopamine into the nucleus accumbens (NA) causing high levels of maternal responding.[13-16] Thus, hormonal stimulation during late pregnancy and birth facilitates the onset of maternal behaviour by increasing infant stimulation of this mPOA-VTA-NA circuit. Although plasticity within this circuit contributes to the maintenance of caregiving behaviour across the postpartum period long after hormonal stimulation has waned,[17] caregiving behaviour likely depends on changes in both antisocial and pro-social neural systems.[18,19] For example, the onset of mothering in rats also coincides with a reduction in the ability of infants to activate fear/defensive neural systems[11] and experimentally induced reactivation of this system can turn mothering off.[20] Thus, the occurrence of caregiving behaviour may depend on both a pup-induced activation of the maternal circuit and an inhibition of a competing fear/escape/attack neural system.[19]

The transition from pup avoidance to pup approach in female rats is typically uni-directional, although male mice can revert back to an aggressive state under certain circumstances. For example, males transition from aggressive or avoidant responses to approach and caregiving responses following sexual experience,[6] whereas, in the absence of continued pup exposure, they can transition back to pup-directed aggression.[21] Therefore, the male mouse model is useful for understanding the relationship between pup-induced activation of a neural system and pup-directed behavioural responses because males engage in aggressive, avoidant or caregiving responses under predictable circumstances. Most of what we know about the relationship between neuronal activity and behavioural response to pups originates from studies that have used immediate early gene (IEG) expression as an indicator of neuronal activity. IEGs are rapidly transcribed and translated in response to an extracellular stimulus because they do not require the de novo synthesis of transcription factors.[22] The protein products of IEGs are transcription factors themselves, which function to regulate the expression of late-responding genes. Note that the pattern of gene expression induced by the same IEG transcription factor can vary greatly by cell.[23] Therefore, although IEG expression is ubiquitous across heterogenous populations of cells, the downstream effects are probably not. Recent work supports the idea that the reduced activation of a central aversion system (including AHN/VMN) in response to pups is also associated with the transition to paternal care.[6,24] Furthermore, expression of the IEG, *cFos*, within the rhomboid part of the dorsal bed nucleus of the stria terminalis (dBNST) was found to be highly correlated with pup-directed aggression,[24] although the mechanism by which activation of a central aversion system mediates distinct types of aversive responses is presently unclear.

The role of pregnancy hormones in activating the maternal neural circuit has been well described, whereas the mechanisms by which these neural systems are activated to promote caregiving behaviour in non-lactating rodents are relatively unknown.[25] Furthermore, how a neural circuit is selected to mediate a specific behavioural response and how factors such as sex, experience or reproductive status regulate the selection of a particular circuit over a competing circuit is unclear. One possibility is that transcriptional patterns within specific cell populations program the activation of a particular circuit. Sex may program a particular circuit for default selection from birth. Reproductive status (sexual experience in males or gestation in females) might re-program the pattern to set a new circuit as default. Repeated experience with pups may lead to neuronal activity-dependent transcriptional changes that result in differential circuit selection (specifically avoidance to approach). Histone deacetylase inhibitor (HDACi) drugs enhance the transcription of genes that are poised or primed for rapid transcription in response to an external stimulus[26] and, in this way, may potentiate experience-driven behavioural modifications. Recently, we found that HDACi treatment in virgin female mice increased the likelihood that regions of the maternal neural circuit, rather than regions of the fear/avoidance circuit, were activated during the challenging task of pup retrieval in a novel T-maze.[27] Based on this finding, we hypothesised that experience-induced changes in behavioural response selection may depend on the extent to which IEGs are primed within neural regions regulating these responses to pups. Furthermore, HDACi treatment may increase the transcription of primed genes that promote the activation and selection of approach circuits exclusively. In the present study, we investigated this hypothesis in pup-naïve virgin male mice because of the considerable variation that they show in their default behavioural response to pups. To assay region-specific transcriptional response to pups, we quantified mRNA expression of two IEGs: *cFos* and neuronal PAS domain protein 4 (*Npas4*).[28,29] We measured *Npas4* in addition to *cFos* because, unlike *cFos*,[30] *Npas4* is exclusively expressed in neurones and is a reliable indicator of neuronal activity.[31] In addition, *Npas4* expression has been shown to be critical for plasticity[32] and the regulation of inhibitory synapse formation on excitatory neurones.[33]

2 | MATERIALS AND METHODS

2.1 | Subjects and drug treatment

All mice were C57BL/6J virgin adult males (≥ 45 days of age) from our breeding colony, naive to pups, housed on a 12:12 hour reversed light/

dark cycle with food and water available ad lib. The HDACi sodium butyrate (Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile water and was administered at a dose of 8 mg mL^{-1} in the drinking water.[34] Control mice received standard drinking water. Drinking water containing sodium butyrate was provided ad lib. beginning 24 hours prior to the start of testing and continued throughout testing. Daily drinking water was monitored for all sodium butyrate-treated mice. All mice were housed individually for 3-7 days prior to and throughout testing. Behavioural testing was conducted 1 hour into the dark phase of the light/dark cycle under dim red light. Stimulus pups were obtained from lactating C57BL/6J or CD1 lactating dams in our donor-pup breeding colony. All procedures were in compliance with the University of California, Davis Institutional Animal Care and Use Committee.

2.2 | Behavioural procedures

2.2.1 | Home cage parental behaviour tests

Pup naïve virgin male mice were treated with sodium butyrate (n = 24) or water (n = 25). Behavioural testing began by scattering three stimulus pups (1-6 days old) in the home cage. Mice were rated using a five-point scale based on their initial response to pups during a 15-minute test: 0 = repeated biting of pups; 1 = rough handled or stepped on pups; 2 = spent less than 50% of the test investigating pups; 3 = spent more than 50% of the test investigating pups; 4 = retrieved at least one pup; and 5 = displayed full paternal care (retrieval, sniffing/licking and hovering over pups). Mice were then categorised based on their score as aggressive (0-1), indifferent (2) or paternal (4-5). None of the mice tested received a score of 3. For male mice that were not aggressive toward pups (scores 1-5), latencies to sniff, retrieve each pup to the nest, sniff/lick the grouped pups and hover over pups in the nest were recorded during the 15-minute test. Pups remained in the cage for a total of 2 hours and were then removed and returned to a lactating dam. In the event that a male attacked a pup, the test was stopped and the pups were immediately removed from the cage. Pups sustaining injuries (visible bite marks, blood or bruising) were euthanised immediately. Male mice that attacked pups on the first test were not tested again. Males that did not attack pups were tested for 2 consecutive days in total.

2.2.2 | Social interaction test

To investigate whether effects of HDACi on behaviour are exclusive to interactions with pups, a separate cohort of pup-naïve virgin male mice treated with sodium butyrate (n = 8) or water (n = 8) was tested in the social interaction test. Sodium butyrate was given beginning 24 hours prior to the start of testing and continued throughout testing. Social interaction testing was conducted in a large Plexiglas open field that contained no bedding (89 × 63 × 60 cm), as described previously.[35] Briefly, the test consisted of three consecutive phases (open field, acclimation and interaction). During the open field phase of testing, each mouse was introduced into the arena for 3 minutes. Time spent in the centre of the arena, corners of the open field and total distance travelled was recorded (Any-Maze; Stoelting, Wood Dale, IL, USA). Following the open field phase, a small wire cage was introduced against one wall of the arena (without removing the focal mouse from the arena). During this 3-minute acclimation phase, the time spent within 8 cm of the novel cage (time investigating novel object) or within the two corners (8 × 8 cm each) opposite the wire cage (time away from novel object) was recorded. During the last phase of testing, an unfamiliar same-sex stimulus mouse was placed into the wire cage for 3 minutes and the time spent investigating the novel mouse was recorded.

2.3 | Region-specific gene expression in aggressive and non-aggressive males

Given that HDACi treatment significantly increased the proportion of animals showing paternal care, but had no effect on the proportion of animals responding aggressively toward pups, we hypothesised that HDACi treatment affects behavioural responses toward pups exclusively in male mice that are not aggressive to pups. To distinguish between the effects of HDACi treatment on activity-dependent gene expression in aggressive vs non-aggressive mice, we pre-screened naïve virgin males for their initial behavioural responses toward pups. A single pup was introduced into the cage and behavioural responses were recorded for 15 minutes. Mice that attacked were categorised as aggressive and mice that failed to attack within the 15-minute test were categorised as non-aggressive. To match the 30-minute pup exposure time between groups at the same time as protecting the pups from infanticide, a wire mesh ball (tea infuser; Norpro, Spring Valley, NY, USA; diameter 1.75 inches) was used with 50 holes (diameter 3 mm). Males could make contact with pups but were not able to injure them. All males were habituated to the presence of the mesh ball prior to testing. Forty-eight hours prior to the start of testing, a mesh ball was placed into each male's cage. The mesh balls remained in the cage until the time of testing at which point the ball was removed and immediately replaced either empty or containing a pup. Gene expression was examined in six groups: pup-naïve virgin male control mice (n = 6), pup-naïve virgin male control mice treated with HDACi (n = 7), aggressive virgin males (n = 7), aggressive virgin males treated with HDACi (n = 7), non-aggressive virgin males (n = 9) and non-aggressive virgin males treated with HDACi (n = 11). On test day, the ball was removed from the cage and replaced with either a pup or no pup (control).

2.4 | Quantification of mRNA by real-time polymerase chain reaction (PCR)

Following 30 minutes of pup exposure, each male was placed in a bell jar containing isoflurane for approximately 15 seconds. To our knowledge, there are no reports of this brief exposure affecting gene

TABLE 1 Taqman primers used for quantitative polymerase chain reactions

Abbreviation	Gene name	RefSeq	Assay ID
cFos	FBJ osteosarcoma oncogene	NM_010234.2	Mm00487425_m1
Npas4	Neuronal PAS domain protein 4	NM_153553.4	Mm01227866_g1
B2m	Beta-2-microglobulin	NM_009735.3	Mm00437762_m1

TABLE 2 Relative expression of *cFos* and *Npas4* normalised to water-treated control mice

Transcript	Brain region	Pup-naïve control (mean ± SEM)	Pup-naïve + HDACi (mean ± SEM)	*P* value
cFos	mPOA	1 ± 0.09	1.03 ± 0.07	0.77
	AHN/VMN	1 ± 0.22	1.10 ± 0.08	0.71
	VTA	1 ± 0.09	0.93 ± 0.24	0.79
	dBNST	1 ± 0.12	0.90 ± 0.09	0.51
Npas4	mPOA	1 ± 0.16	1.28 ± 0.13	0.23
	AHN/VMN	1 ± 0.08	0.72 ± 0.22	0.25
	VTA	1 ± 0.11	1.07 ± 0.12	0.65
	dBNST	1 ± 0.13	1.05 ± 0.13	0.86

Note: In the absence of pup stimulation, there was no effect of histone deacetylase inhibitor (HDACi) treatment on immediate early gene expression (n = 5-6).
Abbreviations: AHN, anterior hypothalamic nucleus; dBNST, dorsal bed nucleus of the stria terminalis; mPOA, medial preoptic area; VMN, anterior hypothalamic nucleus/ventromedial nucleus of the hypothalamus; VTA, ventral tegmental area.

expression and, although it is possible that isoflurane may have produced an effect, experimental and control groups were treated the same. Males were then euthanised by cervical dislocation and brains were immediately removed, frozen and later sectioned (120 μm) on a cryostat and frost-mounted onto slides. The mPOA (bregma 0.37 to −0.35), AHN/VMN (bregma −0.59 to −1.67) and VTA (bregma −2.69 to −3.51) were dissected out using a blunted 15.5-gauge needle and the dBNST (bregma 0.49 to −0.35) was dissected out using a blunted 18-gauge needle sterotaxic.[36] Total RNA was isolated with Qiazol reagent (Qiagen, Valancia, CA, USA) and purified with an RNeasy® Plus Micro Kit (74004; Qiagen) in addition to optional DNase digestion (Qiagen 79254). A Nanodrop™ spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to determine the quality (260/280 ratio > 1.8) and quantity of the RNA. Nine poor quality and 7 off-target samples were not used. The cDNA templates were prepared using a cDNA Synthesis Kit (#4368813; Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. A quantitative real-time PCR was performed using the ABI Viia7 real-time PCR system (Applied Biosystems). The PCR products of interest were detected using TaqMan® Gene Expression assays (Applied Biosystems) (Table 1). All samples were normalised to beta-2 microglobulin (*b2m*). There were no statistically significant differences in the expression of the endogenous control gene between treatment groups. Target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid intrasample variance. All genes of interest were analysed with VIIA7 software (Applied Biosystems) using the comparative cycle thresholds (delta delta CT) method. There were no statistically significant differences in relative gene expression between pup-naïve control mice treated with or without sodium butyrate for any gene tested (Table 2) and therefore these groups were collapsed and the expression of experimental samples was normalised to the average expression of the combined no-pup control group.

2.5 | Serum testosterone assay

To assess whether HDACi treatment could have affected circulating levels of testosterone at the time of pup presentation, a separate cohort of pup-naïve virgin male mice was treated with sodium butyrate (n = 7) or water (n = 7) for 24 hours. Cardiac blood was collected in anaesthetised mice at the time when pups would have been presented (1 hour after lights go off). Blood was left to coagulate at room temperature for ≥ 30 minutes before centrifugation at 3000 *g* for 10 minutes at 4°C. Supernatant was transferred to a clean microcentrifuge tube and stored at −80°C until assayed. A DRG enzyme-linked immunosoarbent assay kit (EIA-1559) (DRG Instruments, Marburg, Germany) was used to assay serum testosterone in accordance with the manufacturer's instructions. The manufacturer reports that the monoclonal antibody has a dynamic range between 0.083 and 16 ng mL^{-1} and the intra assay variance across n = 20 is 4.16%, 3.28% and 3.34% at low, mid and high concentrations, respectively. A standard curve was fit using the four-parameter logistics method. Experimental samples were assayed in triplicate on a single plate and the intra assay variance was 3.41%.

2.6 | Statistical analysis

Probability data were analysed using chi-squared and Fisher's exact tests. The frequency of pup retrieval (number of pups retrieved) was analysed by a mixed two-way one-way analysis of variance (ANOVA) (Treatment × Time), with repeated measures on the second factor. Latency to the first pup contact (sniff) on the first test day was analysed using Student's *t* test because all of the subjects completed the task in the duration of the test. Survival analyses were used to analyse all other latency data (pup retrieval and sniff/lick).[37] This method takes into account that some subjects did not retrieve pups during the 15-minute test and censor those data. These latency data are plotted using Kaplan-Meier survival curves in which the fraction of mice that have retrieved (or sniff/licked) pups at each time point is calculated using the product limit (Kaplan-Meier) method. The Mantel-Cox log-rank test was used to statistically compare survival curves on each test day. In addition, the hazard ratio (HR) and confidence interval (CI) are reported for each variable. The hazard ratio, which is calculated from all the data in the survival curve, indicates the rate at which one group retrieves or licks pups compared to the other. Relative gene expression data were analysed using two-way ANOVA (Behaviour × Treatment). To determine whether IEGs were induced relative to no-pup controls, a one-sample *t* test was used to compare each group to the hypothetical value "1". All other experiments comparing two independent groups were analysed using Student's *t* test. All statistical tests were two-tailed. For ANOVA data, planned comparisons (HDACi vs control within each behaviour) were analysed using Fisher's least significant difference post-hoc tests. All data were analysed using PRISM, version 7 (GraphPad Software Inc., San Diego, CA, USA).

3 | RESULTS

3.1 | Effects of HDACi on the behavioural response to pups

Although the consumption of drinking water was consistent with that reported for C57BL/6J mice,[38] males tended to consume more water if it was treated with sodium butyrate (t_{47} = 6.185, P < 0.0001, η^2 = 0.4487) (Figure 1B). HDACi treatment significantly affected the probability of aggressive, indifferent or paternal responses toward pups

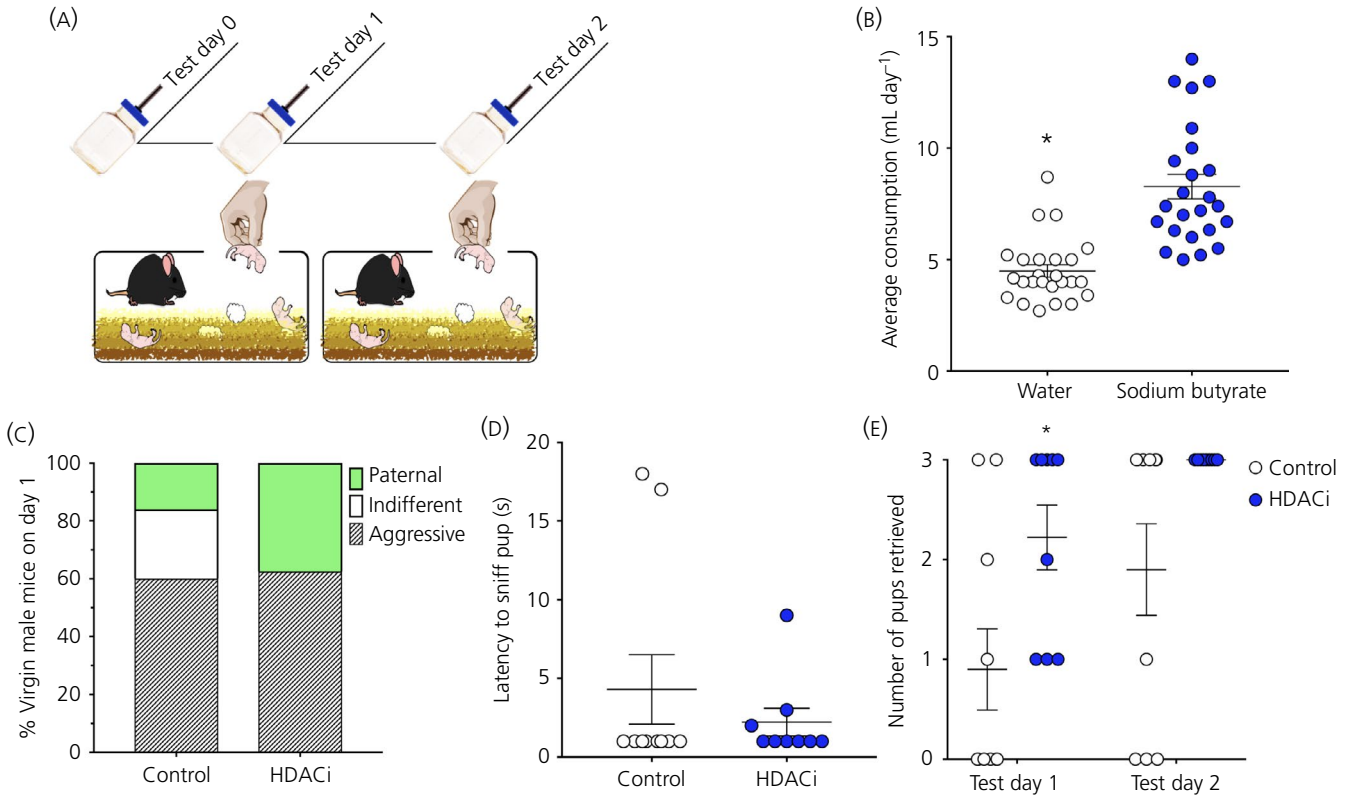

FIGURE 1 Effects of histone deacetylase inhibitor (HDACi) treatment on behavioural response selection in the home cage. A, Timeline for Experiment 1: Mice were treated with HDACi (n = 24) or water (n = 25) for 24 hours prior to the start of testing. B, Males readily consume sodium butyrate-treated water. Average consumption (mL d^{-1}) is represented as the mean ± SEM. *Significantly different from the HDACi group, P < 0.0001. C, Probability of behavioural response to pups varied significantly by treatment (Fisher's exact test, P = 0.02). All non-aggressive HDACi-treated mice showed spontaneous caregiving behaviour compared to 40% of control mice (Fisher's exact test, P = 0.01). D, Mean ± SEM latency to approach and contact a pup on the first test day did not vary by HDACi treatment. NS, not significant. E, HDACi-treated males retrieved more pups than controls and all males showed experience-induced improvements in retrieval (main effects of treatment and time). Only mice that did not show pup-directed aggression were tested on day 2. *Significantly different than control, planned comparison, P < 0.05, d = 1.22

in virgin male mice on the first test day (χ^2 = 7.906, d.f. = 2, P = 0.0192, V = 0.40) (Figure 1C). Specifically, HDACi treatment-induced spontaneous paternal behaviour in non-aggressive male mice (indifferent vs paternal, P = 0.0108, Fisher's exact test). All non-aggressive males retrieved more pups as a result of pup experience (main effect of time: $F_{1,17}$ = 6.434, P = 0.0213, η^2 = 0.12) and HDACi-treated males retrieved more pups than control males (main effect of treatment: $F_{1,17}$ = 10.95, P = 0.0042, η^2 = 0.22), particularly on the first test day (P < 0.05, d = 1.22) (Figure 1E). There were no significant differences in latency to first approach pups on test day 1. The rate of paternal response was significantly affected by HDACi treatment (Table 3). HDACi-treated males were faster to retrieve the first pup on test day 1 (χ^2 = 5.894, d.f. = 1, P = 0.0152, HR = 4.134; 95% CI = 1.314-13.00) (Figure 2). On the second test day, HDACi-treated males were faster to retrieve all pups to the nest (χ^2 = 4.506, d.f. = 1, P = 0.0338, HR = 3.309; 95% CI = 1.096-9.988) and lick pups in the nest (χ^2 = 5.689, d.f. = 1, P = 0.0171, HR = 3.999; 95% CI = 1.280-12.49) compared to control males.

3.2 | Effects of HDACi on social interaction with a novel adult conspecific

There were no significant differences in locomotion (total distance travelled, P = 0.964), thigmotaxis (time in the corners, P = 0.5025) or exploration (time in the centre, P = 0.5256) during the open field phase of the social interaction test (Figure 3). During the acclimation phase, HDACi-treated mice spent more time in the corners (t_{14} = 2.307, P = 0.0369, d = 1.23) and less time investigating the novel empty cage (t_{14} = 2.2448, P = 0.0282, d = −1.31). However, during the social interaction phase of the test, there were no group differences in locomotion (P = 0.3777), thigmotaxis (time in corners, P = 0.4177) or social interaction time (P = 0.6552).

TABLE 3 Hazard ratios, calculated from all the data in the survival curves for pup retrieval and pup licking behaviours, indicate the rate at which histone deacetylase inhibitor (HDACi)-treated males (n = 9) retrieve or lick pups compared to the control males (n = 10)

Measure (HDACi vs control)	Hazard ratio (95% CI)	P value
Test day 1		
Latency to retrieve the first pup	4.13 (1.3-13.0)	0.01*
Latency to retrieve all pups	3.31 (0.7-14.9)	0.12
Latency to sniff/lick pups in nest	2.90 (0.6-12.9)	0.16
Test day 2		
Latency to retrieve the first pup	2.59 (0.9-7.3)	0.07
Latency to retrieve all pups	3.31 (1.1-10.0)	0.03*
Latency to sniff/lick pups in nest	4.00 (1.3-12.5)	0.04*

Abbreviation: CI, confidence interval.
*P < 0.05.

3.3 | Effects of HDACi on circulating testosterone

We tested the possibility that effects of HDACi treatment on spontaneous caregiving behaviour were related to a treatment-induced change in the circulating level of testosterone by assaying plasma testosterone in male mice exposed to sodium butyrate (or regular water) for 72 hours (Figure 4). There was no significant effect of HDACi treatment on testosterone levels in virgin male mice (P = 0.2728).

3.4 | Effects of HDACi on activity-dependent gene expression in approach/avoidance nodes

3.4.1 | *cFos* mRNA expression

cFos expression was significantly induced by pup exposure in all male mice within the mPOA, AHN/VMN and dBNST, regardless of behavioural group or treatment (one-sample t test for each condition in each region, P < 0.05, d_s > 0.5) (Figure 5). *cFos* expression in the VTA was significantly higher in aggressive vs non-aggressive males, regardless of HDACi treatment (main effect of behavioural predisposition: $F_{1,24}$ = 4.762, P = 0.039, η^2 = 0.16). Indeed, in the VTA, pup-induced *cFos* expression failed to reach statistical significance in non-aggressive males compared to an empty mesh ball (P = 0.07, d = 0.75). In the AHN/VMN, there was a significant interaction effect between behavioural predisposition and HDACi treatment in relative *cFos* expression ($F_{1,24}$ = 6.714, P = 0.016, η^2 = 0.22). HDACi treatment reduced *cFos* expression in the AHN/VMN in males that were responsive but not aggressive toward pups (P < 0.05, d = −1.10).

3.4.2 | *Npas4* mRNA expression

The immediate early gene, *Npas4*, was also significantly induced by pup exposure in all male mice regardless of behaviour or HDACi treatment, although only within the mPOA and dBNST, (one-sample t test for each condition in each region, P < 0.05, d > 0.9). Within the VTA, *Npas4* induction was related to behavioural predisposition, with only non-aggressive mice showing a significant elevation of *Npas4* over no-pup control (P < 0.05, d > 0.7). Similarly, induction of *Npas4* by pup-exposure was limited to non-aggressive mice within the AHN/VMN (P < 0.05, d > 0.9). Within the dBNST, behavioural predisposition and HDACi treatment interacted to affect *Npas4* expression ($F_{1,29}$ = 7.569, P = 0.01, η^2 = 0.28). HDACi treatment significantly reduced *Npas4* expression in non-aggressive males (P < 0.05, d = −1.13).

4 | DISCUSSION

Five important conclusions emerge from the results of the present study. First, HDACi treatment-induced spontaneous caregiving behaviour over pup avoidance but had no effect on pup-directed

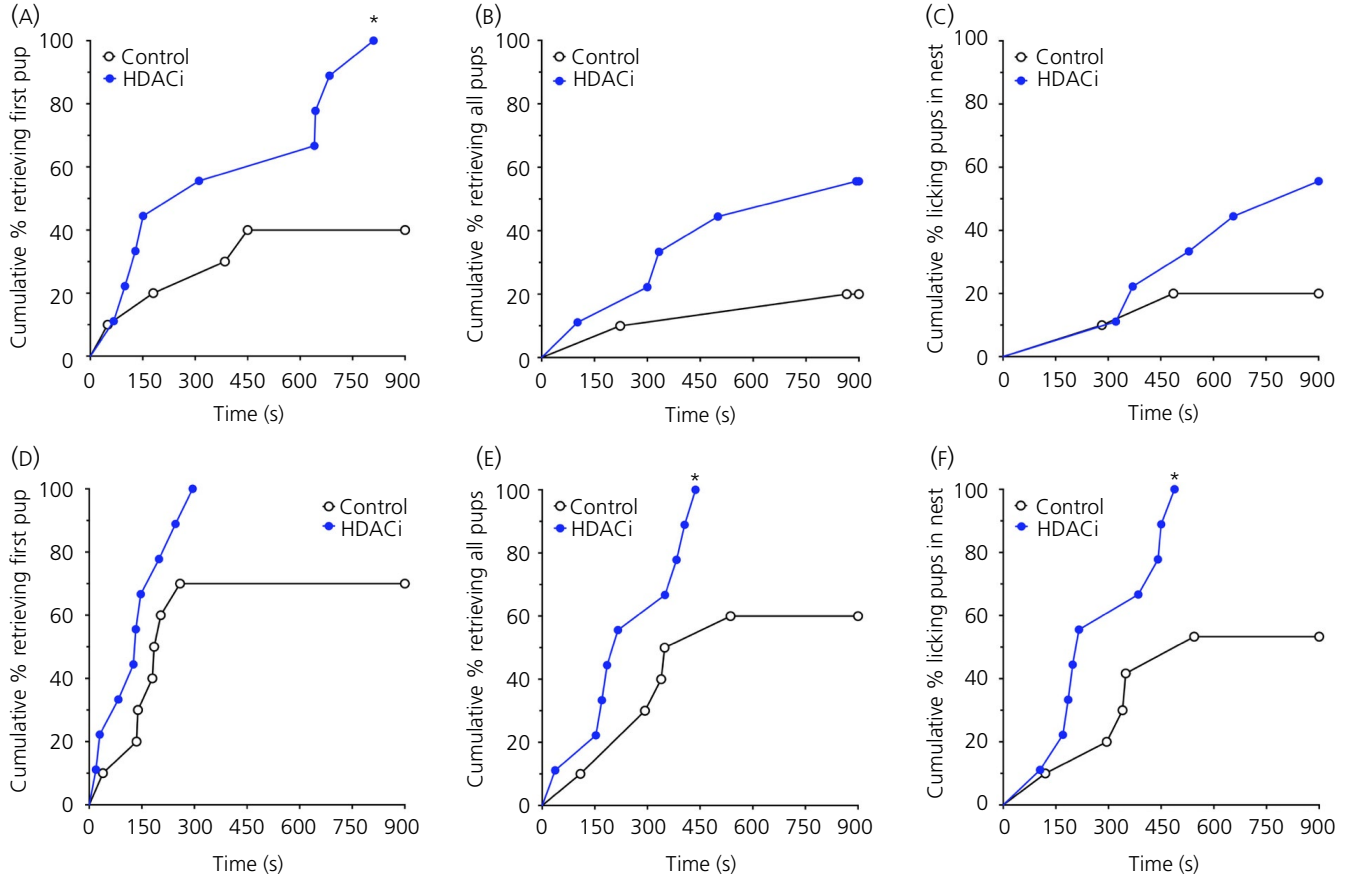

FIGURE 2 Effects of histone deacetylase inhibitor (HDACi) treatment in non-aggressive males on the latency to respond to pups. Kaplan-Meier survival curves show the proportion of animals completing the retrieval tasks (retrieving first or last pup) or licking retrieved pups in the nest at each time point on the *x*-axis in the home cage. A-C, HDACi-treated male mice were faster to retrieve the first pup on test day 1. D-F , HDACi-treated males were faster to retrieve all pups and lick retrieved pups in the nest on test day 2. *Significantly different from control group, chi-squared tests, $P < 0.05$

aggression. For non-aggressive males, HDACi treatment reduced the latency to retrieve the first pup and increased the number of pups retrieved within 15 minutes of the first pup exposure. In addition to its effects on spontaneous care, HDACi treatment also amplified experience-induced changes in caregiving behaviour. HDACi-treated males were faster to group pups and lick pups in the nest compared to non-aggressive controls on test day 2. Second, the pro-social effects of HDACi treatment may be specific to pups because HDACi did not affect social investigation of an adult male conspecific. Furthermore, HDACi treatment did not produce a reduction in general fearfulness as measured by exploration of a novel environment. If anything, HDACi treatment was associated with an avoidance of novel objects. Third, the induction of spontaneous caregiving behaviour by HDACi treatment was probably not related to a reduction in testosterone because HDACi treatment had no significant affect on circulating levels of testosterone. Fourth, in line with the finding that HDACi treatment produces behavioural effects exclusively in non-aggressive mice, the effects of HDACi treatment on IEG expression were also limited to non-aggressive males. For example, *cFos* expression in response to pup cues was reduced in HDACi-treated non-aggressive males within the AHN/VMN. Furthermore, HDACi treatment significantly reduced *Npas4* expression in the dBNST, a region that includes the rhomboid nucleus, which may interfere with caregiving behaviour via its direct inhibition of the central mPOA.[24] By contrast, no effects of HDACi treatment on IEG expression were detected within neural regions associated with pup approach. Both *cFos* and *Npas4* were uniformly induced in the mPOA in all mice exposed to pups. In the VTA, *cFos* expression was induced in mice that show motivated behavioural responses toward pups (regardless of whether that response was pro or antisocial) and, surprisingly, *cFos* was higher in males that showed pup-directed aggression. *Npas4* induction in the VTA, on the other hand, was limited to non-aggressive males. Finally, the two IEGs examined, *Npas4* and *cFos*, did not show the same pattern of expressions in response to the same pup cues in most of the regions examined. Thus, further investigation of *Npas4* in response to pup cues within these circuits may provide new insight into mechanisms of parental care and experience-induced plasticity.

The behavioural results reported in the present study for control-treated male mice are consistent with other reports of the highly variable response of virgin C57BL/6J mice to foster pups.[6] The fact that the facilitatory effects of HDACi treatment on parental behaviour were limited to non-aggressive mice suggests that there

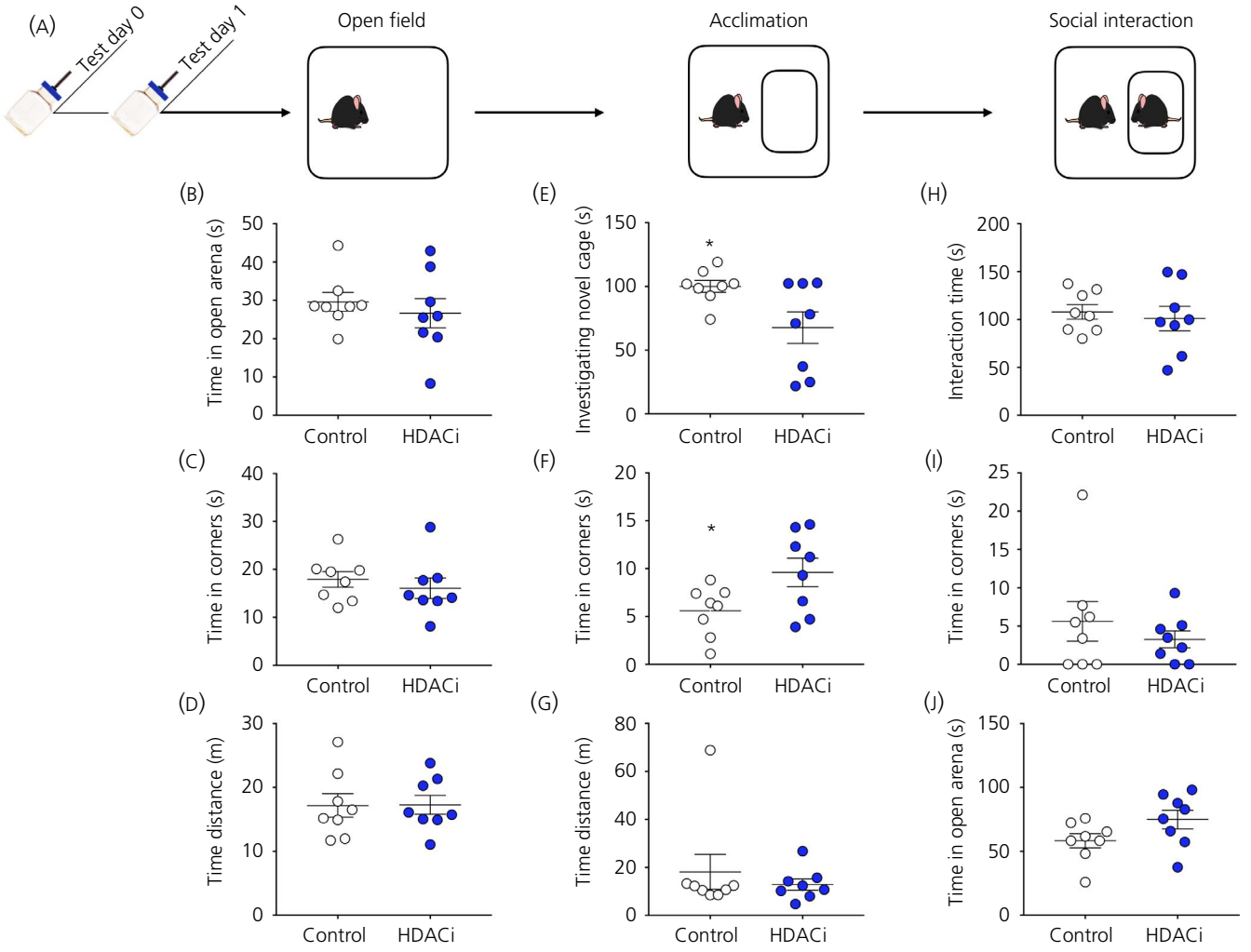

FIGURE 3 Histone deacetylase inhibitor (HDACi) treatment had no effect on social behaviour. A, Timeline for Experiment 2: Males were treated with sodium butyrate in the drinking water or normal water 24 hours before the start of testing (n = 8). The social interaction test consisted of three phases (each lasting 3 minutes). All data are presented as the mean ± SEM. B-D, Activity during the 3-minute open field test was not altered by HDACi treatment. E-G, Upon introduction of a novel empty cage, HDACi-treated males spent significantly more time in the corners of the arena and less time investigating the empty cage. H-J, HDACi treatment had no effect on activity or investigation of a novel adult conspecific. *Significantly different from control group, $P < 0.05$, $d_s > 1.2$

is an interaction between individual variation in response to pups and HDACi treatment. Although there is some evidence for a developmentally regulated onset of aggression in C57BL/6J mice, the factors that contribute to the individual variation in the response of sexually naïve adult male mice to pups are mostly unknown.[39,40] In general, there is weak support for a relationship between circulating testosterone and paternal responsiveness in rodents,[41] although castration does reduce infanticide in virgin male mice.[42] However, in the present study, we found no significant effect of HDACi treatment on circulating levels of testosterone. These data fit with the finding that HDACi treatment also had no effect on aggressive behaviour in virgin male mice.

The finding that HDACi treatment promotes caregiving behaviour exclusively in non-aggressive males is consistent with our previous work, which has reported the facilitatory effects of HDACi in virgin female mice, which are typically non-aggressive.[27,43,44] Taken together, these findings suggest that HDACi treatment acts on a conserved neural substrate. Of course, the extent to which HDACi treatment would fail to promote caregiving behaviour in aggressive female mice is unknown. Note that, when rare instances of infanticide have occurred, we have not found differences between HDACi-treated and control female mice (H.S.M & D.S.S. unpublished data). Although HDACi treatment promotes caregiving behaviour in female and non-aggressive male mice, there is an important inconsistency between its effects in male vs female mice. Our previous work in female mice has emphasised the role of HDACi treatment with respect to enhancing experience-dependent changes in caregiving behaviour. However, the present data suggest that HDACi treatment promotes the initial onset of caregiving behaviour in males. This difference could be related to the fact that the baseline level of maternal responding is much lower in male mice and therefore there is more room to detect a difference between HDACi

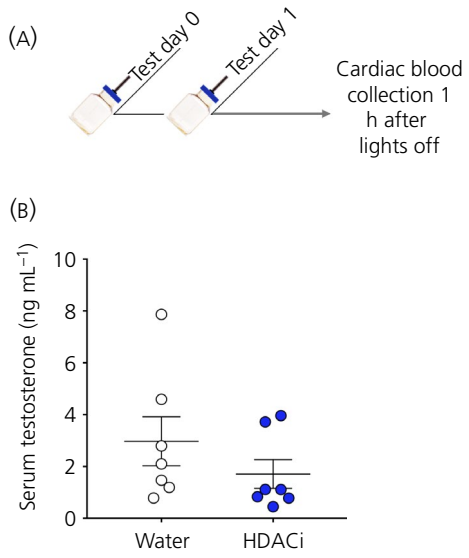

FIGURE 4 Histone deacetylase inhibitor (HDACi) treatment had no effect on serum testosterone. A, Timeline for Experiment 3: Males were given sodium butyrate in the drinking water or normal drinking water for 24 hours prior to cardiac blood collection (n = 7). B, The concentration of testosterone was not significantly different between groups (P = 0.27)

and control groups. However, the fact that HDACi treatment was capable of inducing an onset of caregiving behaviour in some but not all male mice within a few minutes of pup exposure may have implications for the molecular mechanisms by which the drug produces its effect. HDAC inhibitor drugs are highly non-specific.[45] The most commonly used drugs (sodium butyrate, trichostatin A, suberoylanilide hydroxamic acid) inhibit almost all of the HDAC proteins and, because HDACs deacetylate non-histone proteins as well, the effects of these drugs likely extend beyond histone proteins. Despite this, many laboratories, including our own, have reported relatively specific molecular and behavioural effects of HDACi treatment.[43,46] Based on the finding that the distribution of HAT and HDAC proteins is largely overlapping and localised to regulatory regions of genes, one possibility is that HDACi shift the balance of HAT and HDAC activity such that a braking mechanism would be removed from active genes or sequences with active HATs.[26] In this way, stimulus-induced gene transcription would be amplified and therefore fewer experiences with the stimulus might be required for memory consolidation.[47,48] This explanation fits with our previous findings that HDACi treatment reduces the amount of pup experience required to produce long-lasting improvements in maternal care in female mice. However, the finding that HDACi treatment-induced caregiving behaviour on the first trial in a subset of male mice cannot be related to an HDACi amplification of experience-induced gene expression. Furthermore, why would HDACi treatment affect some but not all male mice? We speculate that this differential response to HDACi treatment is related to individual variation in chromatin accessibility. For example, in clinical studies testing the efficacy of HDACi drugs as cancer treatments, the pattern of transcription factor occupancy in cells from individual T-cell lymphoma patients predicts HDACi treatment efficacy.[49] In responsive patients, HDACi treatment is correlated with a rise in DNA accessibility, whereas non-responders show negligible changes in accessibility following treatment. If variation in chromatin accessibility is associated with the differential behavioural response to HDACi treatment in the present study, what might regulate variability in accessibility? One possibility is that genes associated with the maternal responsiveness are poised in non-aggressive males. Poised genes are not active but primed. Multiple mechanisms can mediate this poised or primed state.[50-54] For example, bivalent enhancer sequences are marked by the presence of both the activating (H3K4me1) and repressive (H3K27me3) marks.[50] These sites transition from a poised to active state as a result of a stimulus-induced swap of methylation for acetylation at H3K27.[53,54] Importantly, stimulus-induced *cFos* expression depends on whether RNA pol II is poised at the *cFos* promoter[52]; therefore, the cell-specific pattern of IEG expression in response to pups could depend on which cells have a poised *cFos* promoter. Although we did not address the important issue of cell-specificity in the present study, there is good evidence that pups activate distinct populations of cells in aggressive vs non-aggressive male mice.[55]

In the present study, we examined the expression of two IEG transcripts (*cFos* and *Npas4*) that show stimulus-driven transient expression in the brain. Although *Npas4* expression has never been examined in response to pup cues, *cFos* expression (both mRNA and protein) has been investigated extensively in response to pup cues in both male and female mice, as well as female rats.[6,9,11,24,56-58] Our data indicate a near global induction of *cFos* in response to pup cues, and this finding is consistent with other reports that have identified the mPOA, AHN, VMN and dBNST as regions that are sensitive to pup stimulation. However, the finding that *cFos* induction in these regions was not, for the most part, related to the behavioural response to pups (as determined in the pre-test) was quite unexpected. For example, we hypothesised that *cFos* in the AHN/VMN and dBNST would be exclusively induced in aggressive male mice, whereas *cFos* induction in the VTA and mPOA would be limited to non-aggressive males. Furthermore, we predicted that HDACi treatment would amplify the *cFos* response in the mPOA and VTA of pup-responsive males. These hypotheses were based on previous reports of differential cFos expression in sexually naïve (aggressive) males compared with sexually experienced (paternal) C57BL/6J males. For example, compared to sexually experienced males, aggressive virgins had an exclusive induction of cFos protein in cells of the AHN and ventrolateral VMN, as well as some subregions of dBSNT[6] and, although cFos was induced (relative to non-pup control) in paternal males within some subregions of the dBNST, the cFos response of aggressive males was higher. By contrast, we did not find an exclusive relationship between *cFos* induction in the AHN/VMN and aggressive behaviour. Instead, *cFos* was induced relative to no-pup control in all male mice exposed to pups. However, it should be noted that the present study assayed sexually naïve males with spontaneous aggressive and non-aggressive responses to pups, whereas Tachikawa et al[24] examined paternal males that had experience caring for pups prior to examining pup-induced cFos response. Certainly, virgin

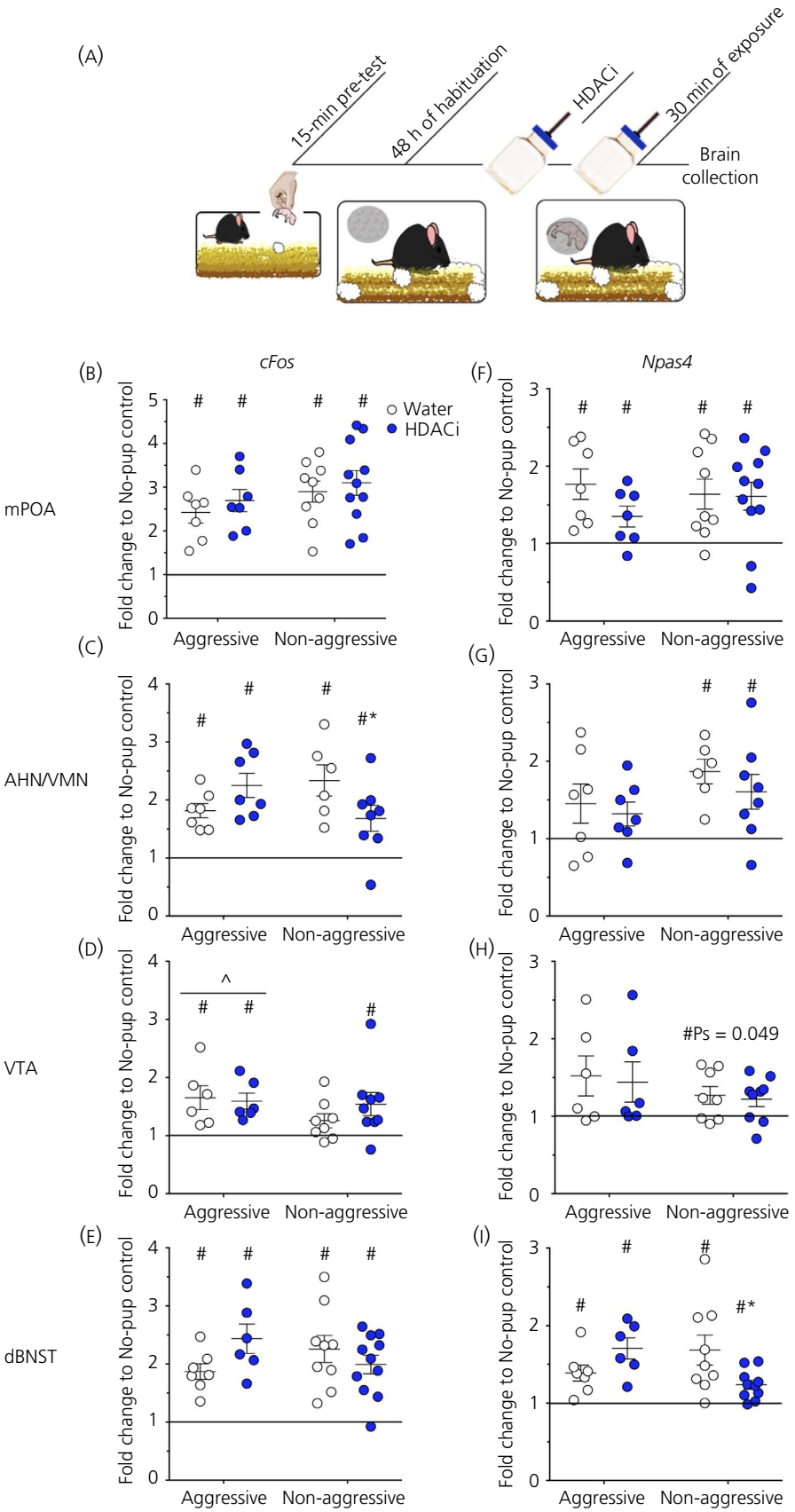
(A)
15-min pre-test
48 h of habituation
HDACi
30 min of exposure
Brain collection
(B)
cFos
(F)
Npas4
mPOA
Water
HDACi
Fold change to No-pup control
Aggressive
Non-aggressive
(C)
(G)
AHN/VMN
(D)
(H)
VTA
#Ps = 0.049
(E)
(I)
dBNST

FIGURE 5 Effects of histone deacetylase inhibitor (HDACi) treatment on immediate early gene (IEG) expression. A, Timeline for Experiment 4: Males were given a brief pre-test to identify aggressive or responsive behaviours toward pups. Following the pre-test, males were habituated to the mesh ball for 48 hours. Twenty four hours prior to pup exposure, males were given HDACi-treated or regular water. On the test day, a pup was placed into the mesh ball for 30 min before brain collection. Mean ± SEM *cFos* (B-E) and Npas4 (F-I) mRNA expression within neural regions associated with pup avoidance/approach (n = 6-11). The black line represents normalisation to the no-pup control group. #Significantly different from no-pup control, one-sample *t* tests, $P < 0.05$. ^Main effect of behaviour, two-way analysis of variance, $P < 0.05$. *Significantly different from the corresponding control-treated group, $P < 0.05$

males showing spontaneous care are less responsive to pups than pup-experienced fathers. Thus, one possibility is that, as caregiving behaviour increases, the ability of pups to induce a cFos response in the AHN/VMN decreases. In support of this idea, males in the present study that would have been most responsive to pups (those treated with an HDACi) did show significantly less *cFos* expression in the AHN/VMN in response to pup cues than non-aggressive controls. Finally, a recent investigation examined pup-induced cFos expression within multiple subregions of the dBNST and mPOA in sexually naïve male mice that were aggressive or spontaneously parental.[24] This work reported that the number of Fos positive cells in the central part of the mPOA and the rhomboid nucleus of the dBNST were highly predictive of paternal or infanticidal responses, respectively. However, when pup cues were presented indirectly (pups presented in a mesh ball), the differences in Fos expression between parental and infanticidal males in all subregions of the mPOA were eliminated and only the rhomboid and anterior lateral parts of the BNST were found to be significantly different between these groups. Our dBNST tissue punches included several subregions of dBNST in addition to the rhomboid/anterior lateral subregions and therefore any effect of the rhomboid region alone may have been washed out.

To our knowledge, the present study is the first to examine differential *cFos* expression in aggressive and responsive virgin males within the VTA. Our hypothesis that non-aggressive males would have higher pup-induced *cFos* expression in the VTA was based on data from virgin female mice.[27] Again, we were surprised to find that *cFos* was induced in both aggressive and non-aggressive HDACi-treated males. Furthermore, aggressive males had significantly higher expression than non-aggressive males, regardless of HDACi treatment. One interpretation is mice that are least likely to approach and interact with pup (non-aggressive control-treated males) do not show a *cFos* response to pup cues in the VTA. Thus, motivation to approach pups, regardless of the intent to kill or care, is associated with *cFos* induction. In support of this idea, optogenetic stimulation of mPOA neurones that project to the VTA increased motivation to reach pups (by climbing over a physical barrier) in male and female mice even though males killed pups once they came into contact with them.[9] Therefore, perhaps it is not surprising that *cFos* expression alone in the VTA does not predict the intention to kill or care for pups. Taken together, these findings fit neatly with the idea that hypothalamic interaction with the mesolimbic dopamine system regulates social motivation more broadly, including approach responses toward both appetitive and aversive stimuli.[59] It should be noted that HDACi-treated non-aggressive males have significantly reduced *cFos* expression in the AHN/VMN coupled with a pup-induced *cFos* response in the VTA, whereas non-aggressive control-treated males have a significantly higher *cFos* response in the AHN but no pup-induced *cFos* response in the VTA. Thus, perhaps it is the combination of these responses that is important for caregiving behaviour.

In addition to *cFos*, we chose to examine *Npas4*, another IEG with a similar time course of induction to *cFos*.[32] Although *cFos* transcription is induced in brain cells by a number of different extracellular stimuli, *Npas4* induction is specifically linked to depolarisation of neurones and therefore may provide some indication of the neuronal response to pups within these regions.[33] Furthermore, *Npas4* expression is induced in response to learning, rather than exposure to novel or robust stimuli. For example, *Npas4* is induced in the hippocampus following contextual fear learning but, unlike *cFos*, *Npas4* expression is not induced by shock alone.[32] Once translated, Npas4 protein serves as a transcription factor, regulating the expression of several late-responding genes that are also critical for neuronal plasticity and particularly new synapse formation.[29] Thus, stimulus-induced expression of *Npas4* might suggest a neuronal response to pups rather than an increased input to cells as a result of pup exposure. Our data indicate that *Npas4* induction was limited to non-aggressive males in both the AHN/VMN and VTA, although the data from the VTA may be interpreted with some caution because this result barely reached statistical significance. HDACi treatment was without effect on *Npas4* expression in these sites; thus, *Npas4* induction in these regions might be linked to the non-aggressive behavioural response rather than caregiving behaviour per se. With respect to HDACi-induced changes in *Npas4* expression, the dBNST was the only site affected. Therefore, the HDACi-induced reduction in *Npas4* expression may be related to the induction of paternal care. Finally, the dBNST and the mPOA may be particularly sensitive to pup stimuli given that we found a significant induction of both *Npas4* and *cFos* in all males exposed to pups. The fact that HDACi treatment significantly lowered *Npas4* in the dBNST fits with the idea that this region plays an inhibitory role in parental behaviour, although the present data are not consistent with the possibility that this role involves the exclusive regulation of pup-directed aggression.

In conclusion, the results of the present study indicate that HDACi treatment can induce spontaneous caregiving behaviour in non-aggressive male mice. The facilitatory effect of HDACi treatment is robust and specific to parental behaviour. All non-aggressive males with HDACi treatment responded to pups within 15 minutes of pup exposure and HDACi treatment did not reduce neophobia or increase social behaviour generally. HDACi-induced reduction in IEG expression within two sites that inhibit caregiving behaviour is likely related to the induction of spontaneous caregiving behaviour; however, the

overall pattern of pup-induced IEG expression was not entirely supported by our predictions. An aggressive behavioural predisposition was not associated with the exclusive expression of *cFos* in regions of the brain linked to fearful/defensive behaviour in response to pup cues. Similarly, we did not find greater activation of IEG expression in the mPOA of non-aggressive males in response to pup cues. Taken together, these findings emphasise the importance of understanding how the mPOA and its interaction with downstream neural sites regulate spontaneous care, indifference or pup-directed aggression. Finally, the present data support the idea that *Npas4* expression may be a more specific marker for neuronal activation because, unlike *cFos* expression, *Npas4* was differentially expressed in non-aggressive and aggressive mice. Future work will need to gain a cellular resolution of *Npas4* activity in these regions aiming to better understand its role in paternal experience-induced plasticity.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

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