Early life stress influences acute and sensitised responses of adult mice to cocaine by interacting with GABA$_A$ α2 receptor expression

Claire I Dixon[1], Sophie E Walker[1], Jerome Swinny[2], Delia Belelli[3], Jeremy J Lambert[3], Sarah L King[1] and David N Stephens[1]

[1]Laboratory of Behavioural and Clinical Neuroscience, School of Psychology, University of Sussex, Brighton, UK,

[2]Department of Pharmacy, University of Portsmouth, Portsmouth, UK

and

[3] Centre for Neuroscience, University of Dundee, Dundee, UK

Address for Correspondence:

Prof D N Stephens,

School of Psychology,

University of Sussex,

Falmer,

Brighton BN1 9QH

+44 1273 678485 or +44 7802 378413

d.stephens@sussex.ac.uk

Abstract

Early life stress (ELS) is known to exert long term effects on brain function, with resulting deleterious consequences for several aspects of mental health, including the development of addiction to drugs of abuse. One potential mechanism in humans is suggested by findings that ELS interacts with polymorphisms of the *GABRA2* gene, encoding $\alpha 2$ subunits of $GABA_A$ receptors, to increase risk for both posttraumatic stress disorder, and vulnerability to cocaine addiction. We used a mouse model, in which the amount of material for nest building was reduced during early postnatal life, to study interactions between ELS and expression of $\alpha 2$-containing $GABA_A$ receptors in influencing cocaine-related behaviour. Breeding of parents heterozygous for deletion of $\alpha 2$ resulted in litters containing homozygous knockout ($\alpha 2^{-/-}$), heterozygous knockout ($\alpha 2^{+/-}$), and wildtype ($\alpha 2^{+/+}$) offspring. Following the ELS procedure, the mice were allowed to develop to adulthood before being tested for the acute effect of cocaine on locomotor stimulation, behavioural sensitisation to repeated cocaine, and to cocaine-conditioned activity. Exposure to ELS resulted in increased acute locomotor stimulant effects of cocaine across all genotypes, with the most marked effects in $\alpha 2^{-/-}$ mice (which also showed increased activity following vehicle). Repeated cocaine administration to non-stressed mice resulted in sensitisation in $\alpha 2^{+/+}$ and $\alpha 2^{+/-}$ mice, but, in keeping with previous findings, not in $\alpha 2^{-/-}$ mice. Prior exposure to ELS reduced sensitisation in $\alpha 2^{+/+}$ mice, albeit not significantly, and abolished sensitisation in $\alpha 2^{+/-}$ mice. Conditioned activity was elevated following ELS in all animals, independently of genotype. Thus, while the enhanced acute effects of cocaine following ELS being most marked in $\alpha 2^{-/-}$ mice suggests a function of $\alpha 2$-containing $GABA_A$ receptors in protecting against stress, the interaction between ELS and genotype in influencing sensitisation may be more in keeping with ELS reducing expression of $\alpha 2$-containing $GABA_A$ receptors. The ability of ELS to increase cocaine-conditioned locomotor activity appears to be independent of $\alpha 2$-containing $GABA_A$ receptors.

Key Words: addiction; knockout; conditioned activity; behavioural sensitisation;

Introduction

Considerable evidence associates variations in the *GABRA2* gene encoding the $\alpha 2$ subunit of GABA$_A$ receptors with the development of substance abuse disorders (see (Stephens et al., 2017) for a review). Thus, in human populations, Edenberg and colleagues (Edenberg et al., 2004) were the first to identify two haplotype blocks extending downstream from intron 3 within *GABRA2*, with the more common haplotype being a significant risk factor for alcohol dependence. Subsequently, other workers have reported supportive evidence (e.g. (Soyka et al., 2008), a more discrete association for only those alcoholics with concurrent illicit substance disorder (Agrawal et al., 2006), or with more general conduct disorders (Dick et al., 2006, Melroy et al., 2014). Nevertheless, the evidence is complex, and other researchers have found associations of *GABRA2* variants with substance abuse disorders that differ from these reports (e.g. (Covault et al., 2004, Lappalainen et al., 2005, Fehr et al., 2006), possibly indicating differing types of alcoholism, including those associated with high *vs.* low anxiety (Ducci et al., 2007, Enoch, 2008). Similar (though less extensive) findings suggest related associations of GABRA2 variants with cannabis abuse (Agrawal et al., 2008b), nicotine dependence (Agrawal et al., 2008a), and cocaine addiction (Dixon et al., 2010, Enoch et al., 2010). The Enoch et al (2010) study is particularly interesting as the association was strongest in individuals who had suffered childhood trauma, raising the possibility that *GABRA2* variants may contribute to the development of addictions by increasing vulnerability to early life stress, a factor known to contribute to adult addictive behaviour (Enoch, 2011, Cadet, 2016, Messina et al., 2008). Consistent with that idea, polymorphisms in *GABRA2* interact with early childhood trauma in increasing risk for PTSD (Nelson et al., 2009).

The possibility that GABRA2 variations contribute to vulnerability to early life stressful events is plausible. It has been known for some time that the anxiolytic action of benzodiazepines and barbiturates in rodents depends on their facilitation of transmission at GABA$_A$ receptors expressing $\alpha 2$ subunits, the protein encoded by *Gabra2* (Dias et al., 2005, Dixon et al., 2008, Low et al., 2000, Morris et al., 2006, Smith et al., 2012). More relevant to the current context, loss of $\alpha 2$-subunit expression leads to increased responsiveness to threatening events (Dixon et al., 2008), suggesting increased susceptibility to anxiety. Thus, we might speculate that impairment of transmission at $\alpha 2$-containing receptors may decrease resistance to stressful events in childhood, leading to more severe consequences for adult behaviour, including

vulnerability to substance abuse. Alternatively, there is evidence from rodent studies (Caldji et al., 2000, Hsu et al., 2003, Skilbeck et al., 2017, Mitchell et al., 2018) that early life stress itself affects expression levels of GABA$_A$ receptor subunits, especially α2 subunits (Mitchell et al., 2018). Importantly, these effects may be regionally different, and depend on the early life stress procedure used. Although, to date, there is little evidence to support either hypothesis in humans, it is worth noting that post-mortem studies of brains of alcoholic patients found lowered α2-subunit mRNA in the central nucleus of the amygdala (Cammarota et al., 1995). Further, expression of α2 mRNA is reduced in human neural cell cultures expressing the *GABRA2* haplotype associated with addictions (Lieberman et al., 2015).

We therefore investigated potential interactions between early life stress and expression of the Gabra2 gene in a mouse model, employing disrupted mother-pup interactions, in which we have previously shown that deletion of the gene encoding α2 subunits alters behavioural adaptations to cocaine (Dixon et al., 2010, Mitchell et al., 2018). A number of studies have already shown that early life stress alters both acute and sensitised responses to psychostimulants (Brake et al., 2004, Kikusui et al., 2005, Li et al., 2003, Marin and Planeta, 2004, Mitchell et al., 2018). In the current report, we extended our previous findings by breeding mice from parents heterozygous for deletion of α2 (α2$^{+/-}$), so that the resulting litters contained a mixture of α2$^{+/+}$. α2$^{+/-}$, and α2$^{-/-}$ offspring, but ensuring that the mother's genotype did not contribute differentially to the stress manipulation (*i.e.,* our previous study bred either α2$^{+/+}$ or α2$^{-/-}$ parents to derive wildtype and knockout offspring, leaving open the possibility that the procedure gave rise to different levels of stress in the wildtype and knockout mothers. Indeed, we reported that α2$^{-/-}$ mothers showed altered mothering relative to wildtype mothers even under non-stress conditions (Mitchell et al., 2018). We then exposed half of the litters to early life stress (ELS) by reducing the bedding material available for nest building for 7 days, before rearing the mice to adulthood under standard conditions, at which time they entered behavioural testing. We predicted that mice exposed to ELS would show altered ability to sensitise to cocaine in adulthood, and that this change would be influenced by level of α2-expression.

Materials and Methods

Animals

α2-subunit knockout ($\alpha 2^{-/-}$) mice were generated as previously described (Dixon et al., 2008) and have since been maintained on a mixed 50% C57BL/6J –50% 129SvEv background at the University of Sussex animal facility. Heterozygous pairings of virgin animals were used (n=80 pairs) in order to produce wildtype ($\alpha 2^{+/+}$), heterozygous ($\alpha 2^{+/-}$) and knockout ($\alpha 2^{-/-}$) mice for experiments. Dams were single-housed except for a three-night mating period during which the sire was introduced.

Early-life stress (ELS) model

We replicated a procedure in which nesting material is reduced for one week during the early post-natal period. This model serves to disrupt the dam-pup interaction and is associated with several stress-related perturbations in adult mice (Rice et al., 2008). Once pregnancy was detected, two weeks after removal of the sire, individual cages were randomly assigned to either the control or stress condition. Cages were discretely monitored every 12 hours for the birth of pups. The day of birth was termed postnatal day (p) 0. On the morning of p2 litters were adjusted to six pups, at least one of which was female.

To initiate the stress procedure, dams and their litters were moved to clean cages (33 x 15 x 13cm). Control dams (n=37) were provided with a standard amount of sawdust (≈650 ml) and nesting material (one square of compressed cotton material measuring 5 × 5 cm (Nestlet, Ancare, Manchester, UK). Stress dams (n=43), were provided with a reduced amount of bedding: Sawdust (≈50ml) was spread underneath a fine-gauge aluminium mesh platform, raised 1cm from the cage floor (0.4 x 0.9 cm mesh, Rudgewick Metals Ltd, Sussex, UK). One half of a Nestlet (5 x 2.5 cm) was provided additionally, placed atop the mesh. Litters remained undisturbed from p2 to p9. On p9 all litters were weighed and placed in fresh cages of a standard, control set-up. These cages were changed again once before weaning, which occurred at p21.

At weaning, genotyping was carried out. Following amplification by PCR, the presence of DNA was determined by gel electrophoresis, using primers for wildtype and knockout versions of the Gabra2 gene. Since the experimental stress manipulation was of the litter, each pup

within a litter must be considered a replicate (Abbey and Howard, 1973). For this reason, a maximum of one mouse per genotype per litter went forward into each experiment (some litters did not contain all three possible genotypes).

Experimental animals were housed in pairs or triplets. All animals were housed under a 12 hr light/dark cycle (lights on at 07:00) in a holding room with controlled temperature (≈21°C) and humidity (≈50%). Animals had *ad libitum* access to standard laboratory chow (Bekay Feeds, Hull, UK) and water within the home cage. All experiments were approved by the institutional ethics committee and were performed under United Kingdom legislation on animal experimentation (Animal [Scientific Procedures] Act, 1986) following ethical approval. All experiments took place between 08:00 and 13:00.

Behavioural sensitisation to cocaine

Male mice (n=157; 10 weeks old at the start of the experiment) were divided into 12 groups according to their early-life experience, genotype and the treatment group to which they had been assigned. Where a single litter (either ELS or control) contained more than one male from a particular genotype, a single representative of that genotype was chosen at random. This gave rise to the groups described in Table 1. Mice of different genotype and stress history were randomly allotted to each piece of apparatus and to time of testing within a day, both of which, however, remained the same for individual animals across testing days.

Apparatus

Locomotor activity was assessed using 16 black Perspex, circular runways (internal diameter, 11 cm; external diameter, 25 cm; height, 25 cm) atop a translucent platform. Illumination of the runways was achieved by 2 fluorescent tubes (T4, 30 watt) positioned above a translucent Perspex sheet suspended 20 cm above the runways. Animals were videoed from below through a translucent Perspex floor by a camera (Fire-i; UniBrain, Scorpion Vision Software, Hampshire, UK) that detected the moving shadow of the animal. Images were digitised, recorded and locomotor activity determined using in-house software written in Matlab (The MathWorks, Cambridge, UK). Overall distance traveled (m) was calculated.

Drugs

Cocaine hydrochloride (Macfarlan Smith, Edinburgh, UK) was dissolved in 0.9% saline. Injections were administered at a volume of 10 ml/kg i.p.

Procedure

Mice were habituated to the circular runways in two, once-daily, sessions. Animals were removed from the apparatus after 30 minutes, returned to the home cage for 5 minutes (during which the apparatus was cleaned), and then placed in the runways again for a further 60 minutes. On the third day, following the 30 minute habituation session, mice were removed from the circular runways and were injected with vehicle (10 ml/kg saline i.p.) before being placed in the runway again. Activity was recorded for a further 60 min post-vehicle injection.

Mice then received repeated, intermittent injections of 10 mg/kg cocaine for 10 sessions, across 12 days (Monday-Friday, with a 2-day break at the weekend). Similarly to the habituation sessions, mice were habituated to the circular runways for 30 min, removed and then injected with cocaine. Activity was recorded for a further 60 min post-cocaine injection.

Conditioned Activity

Three days after the final sensitisation session, mice were habituated to the circular runways for 30 min, removed and then injected with vehicle. Activity was recorded for a further 60 min post-vehicle injection.

Statistics

Pup mortality under stress and non-stress conditions was compared using Chi-square tests. Body weights in the stress and non-stress conditions were compared at p9 and p21 (before genotyping) using Student t-tests, while 2-way analysis of variance (ANOVA) was used to assess consequences of stress vs. non-stress conditions, and genotype at p70. Acute responses to cocaine and conditioned activity were assessed by 3-way ANOVA, with early-life experience (control, stress), genotype (WT, HT, KO) and drug treatment (saline, cocaine) as between-subject factors. Development of behavioural sensitisation to cocaine was assessed *via* 4-way, mixed-factor ANOVA, with the same between-subject factors and the additional repeated measure of session (day 1 vs day 10 of cocaine treatment). Where significant (p ≤

0.05) main effects or interaction terms were found, further analysis was performed using ANOVA and individual between- or within-genotype comparisons by *t*-test.

Results

Early life stress effects.

Pup mortality. The mortality rate of pups was not significantly different between early-life stress (ELS) and non-stress breeding conditions (Fig. 1; $\chi^2_{(1, n = 450)}$ = 0.977, p = 0.323), with deaths at 1.93% and 0.86%, respectively.

Pup weight. Pups subjected to the ELS condition were significantly lighter in body weight at p9 (t(46) = 6.686, p < 0.001) and p21 (t(46) = 2.935, p < 0.01; Fig. 1A) than non-stressed controls. At p70 (Fig. 1B), stressed mice weighed significantly less than their non-stressed counterparts (main effect of ELS: $F_{(1,150)}$ = 11.218, p < 0.001). Whilst KO mice weighed less than WT and HT (main effect of genotype: $F_{(2,150)}$ = 22.981, p < 0.01; WT vs. KO: t(95) = 6.409, p < 0.001; HT vs. KO: t(103) = 5.457, p < 0.001), this was independent of the ELS condition implemented (non-significant ELS by genotype interaction: $F_{(2,150)}$ = 0.252, p = 0.777).

Behavioural sensitisation to cocaine

Habituation. During habituation to the apparatus and injection procedure, all mice decreased their locomotor activity (Fig. 2; main effect of session: $F_{(2,302)}$ = 73.768, p < 0.001). However, KO mice showed an increase in locomotor behaviour in response to the sham saline injection on session 3 (session by genotype interaction: $F_{(4,302)}$ = 8.236, p < 0.001).

Acute cocaine response. On the first day of sensitisation, the locomotor response was higher in ELS compared to non-stressed animals (Fig. 2B; main effect of ELS: $F_{(1,145)}$ = 22.753, p < 0.001; ELS by drug interaction: $F_{(1,145)}$ = 23.510, p < 0.001). The increased locomotion was evident in response to cocaine administration (main effect of ELS: $F_{(1,76)}$ = 22.466, p < 0.001) but not in vehicle treated groups (main effect of ELS: $F_{(1,77)}$ = 0.083, p = 0.774). Locomotor activation was also higher in $\alpha2^{-/-}$ animals (main effect of genotype: $F_{(2,145)}$ = 6.196, p < 0.01; Bonferroni *post hoc* comparison: p < 0.05 compared to $\alpha2^{+/+}$), but this effect was independent of drug treatment or ELS condition (ELS by genotype interaction: $F_{(2,145)}$ = 0.498, p = 0.609; genotype by drug interaction: $F_{(2,145)}$ = 1.880, p = 0.156). No differences in locomotor activity

between $\alpha2^{+/-}$ and $\alpha2^{+/+}$ following acute drug or vehicle administration were seen (Bonferroni *post hoc* comparison: p >0.05 compared to $\alpha2^{+/+}$).

Sensitisation to cocaine. The effects of ELS and genotype on repeated locomotor activity differed according to drug administration (Fig. 3A; session by ELS by drug interaction: $F_{(9,1305)}$ = 3.778, p < 0.001; session by genotype by drug interaction: $F_{(9,1305)}$ = 3.008, p < 0.001). Repeated saline administration resulted in a significant habituation (main effect of session: $F_{(9,657)}$ = 5.748, p < 0.001) which did not differ according to either stress condition or genotype (day by ELS by genotype interaction: $F_{(18,657)}$ = 0.446, p = 0.977). Repeated cocaine administration resulted in a significant sensitisation (main effect of session: $F_{(9,648)}$ = 3.771, p < 0.001), which was reduced by prior exposure to ELS (ELS by session interaction: $F_{(9,648)}$ = 3.605, p < 0.001; main effect of ELS: $F_{(1,72)}$ = 9.724, p < 0.01). The degree of sensitisation was altered according to genotype (genotype by session interaction: $F_{(18,648)}$ = 3.374, p < 0.001), reflecting the previously reported absence of sensitisation in $\alpha2^{-/-}$ mice. However, any genotype effects observed were not altered by exposure to the ELS paradigm (ELS by genotype by session interaction: $F_{(18,648)}$ = 1.028, p = 0.425).

In order to test how exposure to ELS changed the degree of sensitisation, a measure of sensitisation was calculated by subtracting the acute response to cocaine on day 1 from the sensitised response on day 10 (Fig. 3B). The degree of sensitisation observed was sensitive to manipulations of ELS and genotype (ELS by genotype by drug interaction: $F_{(2.157)}$ = 3.621, p < 0.05), which were present in cocaine treated groups (ELS by genotype interaction: $F_{(2.78)}$ = 3.310, p < 0.05) but not vehicle-treated animals (ELS by genotype interaction: $F_{(2.79)}$ = 0.317, p = 0.729). Exposure to ELS did not affect the degree of sensitisation in $\alpha2^{+/+}$ ($t_{(24)}$ = 0.561, p = 0.132) or $\alpha2^{-/-}$ mice ($t_{(22)}$ = -0.146, p = 0.885), but ELS significantly reduced the expression of sensitisation in $\alpha2^{+/-}$ mice ($t_{(26)}$ = 3.838, p < 0.01).

Conditioned activity. The presence of conditioned activity was confirmed following the sensitisation procedure in a single session in which all mice received an injection of saline vehicle. An increased locomotion in animals previously treated with cocaine was found when compared to those previously treated with vehicle (Fig. 4; main effect of drug: $F_{(1,145)}$ = 24.329, p < 0.001). This effect was greater in stressed animals (main effect of ELS: $F_{(1,145)}$ = 5.981, p <

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0.05; ELS by drug interaction: $F_{(1,145)} = 7.174$, $p < 0.05$) and occurred to the same extent in all genotypes (ELS by drug by genotype interaction: $F_{(2,145)} = 0.187$, $p = 0.829$).

Discussion

Our experiments reveal three different patterns of interaction between early life stress and GABA$_A$ α2 receptors in determining responses to cocaine. Firstly, in keeping with our recent report (Mitchell et al., 2018), exposure to ELS resulted in increased acute effects of cocaine in stimulating locomotor activity in all genotypes. A similar increase in responsiveness to the acute effects of cocaine has also been reported in male (but not female) mice following a different method of inducing early life stress, repeated maternal separation (Kikusui et al., 2005), and in rats (Marin and Planeta, 2004). Similarly, another psychostimulant, amphetamine, given acutely induced a greater stimulant effect in adult rats previously subjected to ELS (Brake et al., 2004). On the other hand, others (Gracia-Rubio et al., 2016, Li et al., 2003) found no effects of maternal separation on responsivity to the acute locomotor stimulant effects of cocaine. The extent to which these different results reflect different methods of inducing early life stress is unclear.

In our experiment, ELS enhanced the acute effects of cocaine in all genotypes, suggesting that baseline levels of α2 receptors do not interact with stress to alter acute cocaine locomotion. Previous data have suggested that α2$^{-/-}$ mice are more sensitive to the acute locomotor effects of cocaine in stress conditions (Mitchell et al., 2018) . However, in the Mitchell *et al* experiments, ELS and α2$^{-/-}$ mice were tested independently in separate experiments, so that a direct comparison is not possible. In the current study, however, α2$^{-/-}$ mice were more sensitive to the effect of vehicle injection (no genotype x drug interaction), and also to a sham injection during the habituation procedure. That mice with lowered expression levels of the *Gabra2* gene may be more sensitive to stressful situations is consistent with our previous report of increased reactivity of α2$^{-/-}$ mice to a signal predicting shock (Dixon et al., 2008), and in keeping with our hypothesis. Interestingly, no difference was seen between heterozygous and wildtype mice, perhaps suggesting that a partial loss of α2-containing receptors is insufficient to influence cocaine sensitivity. (Note, accumbens *Gabra2* mRNA is reduced by approximately 50% in the heterozygous animals (Dixon et al., 2010), but we have no direct evidence whether number of α2-containing receptors was reduced).

The consequences of ELS for cocaine sensitisation were more complex. Repeated cocaine administration resulted in sensitisation and this was reduced by prior exposure to ELS (main effect of ELS), in keeping with both our previous work (Mitchell et al., 2018) and a previous

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report that ELS attenuated sensitisation in adulthood (Gracia-Rubio et al., 2016). Similarly, amphetamine, given acutely, induced a greater stimulant effect in adult rats subjected to ELS, but, whereas the amphetamine response showed sensitisation in controls, no such sensitisation was seen in the rats subjected to ELS (Brake et al., 2004). The extent to which an elevated acute response to cocaine following ELS masked subsequent sensitisation is not clear, though, in our study, the level of activity in the sensitised mice was certainly not at ceiling. However, in this study (and in contrast to (Mitchell et al., 2018)) there were no significant effects of ELS on sensitisation in either WT or $\alpha 2^{-/-}$ mice. (Although ELS reduced the degree of sensitisation in WT mice, in the present study the effect was not significant). Most importantly, ELS abolished sensitisation in the heterozygous $\alpha 2^{+/-}$ mice, which had demonstrated a normal response to acute cocaine. Thus, in this respect, ELS impaired behavioural sensitisation in a way reminiscent of the effects of deletion of $\alpha 2$ (Dixon et al., 2010).

From the present behavioural data set, the interpretation of these findings remains speculative. Since $\alpha 2^{-/-}$ mice in the control, non-stress condition failed to show sensitisation, there was no opportunity for ELS to show an additional effect in the knockout mice. On the other hand, $\alpha 2^{+/-}$ mice in the non-stress control group did not differ from WT controls in their response to repeated cocaine, suggesting that a partial loss of receptors was insufficient to give rise to altered response to cocaine. Nevertheless, following ELS exposure, $\alpha 2^{+/-}$ mice resembled $\alpha 2^{-/-}$ mice in that they no longer showed sensitisation, consistent with ELS effects adding to those of a partial loss of $\alpha 2$ receptors in the $\alpha 2^{+/-}$ animals (Dixon et al, 2010). Thus, the non-significant decrease in sensitisation in the $\alpha 2^{+/+}$ mice following ELS might be consistent with that ELS reducing $\alpha 2$ expression, but still not sufficiently to significantly influence this behaviour.

In keeping with that hypothesis, we previously reported that ELS exposure results in a long-lasting reduced expression and function of $\alpha 2$-containing GABA$_A$ receptors in nucleus accumbens core (Mitchell et al., 2018). While during rodent late embryonic development $\alpha 2$-containing GABA$_A$ receptors are the predominant species, during early postnatal development $\alpha 1$-containing receptors become predominant (Fritschy et al., 1994). It is thus plausible that ELS may affect this developmental switch, resulting in reduced $\alpha 2$ levels.

However, others, using a different form of early life stress, have suggested an increased level of expression of $\alpha 2$ in other brain areas (amygdala and medial prefrontal cortex) (Gondre-Lewis et al., 2016). In keeping with that observation, brief (15 min) early life handling between P1 and P14 has been suggested to protect against the normal developmental replacement of $\alpha 2$ with $\alpha 1$ subunits, at least in cortical and thalamic areas (Skilbeck et al., 2017). Skilbeck et al. (2017) argue that their early-handling model provides a stress-protective effect, since such brief interventions can result in increased mother-pup interactions. In contrast, our procedure results in increased sorties of the mother out of the nest (Mitchell et al., 2018), accompanied by reduced licking, grooming and arched-back nursing, thereby inducing a more severe form of mother-pup separation (Rice et al., 2008). Thus, a potential explanation of the current behavioural data is that ELS influenced sensitisation by reducing $\alpha 2$ expression; while, in our experiment this effect was insufficient in $\alpha 2^{+/+}$ mice to show a reliable effect on sensitisation, when ELS was combined with a presumed partial loss of $\alpha 2$ receptors in the heterozygous mice, the additive loss gave rise to a behavioural effect resembling that seen in $\alpha 2^{-/-}$ mice. (Note, in parallel experiments from our groups, but in a different animal unit, and using mildly different stress parameters, ELS prevented sensitisation in WT mice (Mitchell et al., 2018)).

The third observation of interest is that conditioned activity was elevated in all ELS groups, regardless of genotype, while deletion of $\alpha 2^{-/-}$ did not increase this conditioned behaviour. This finding implies an increased strength of conditioning following ELS that is independent of $\alpha 2$. In agreement, in no previous study have we have seen effects of manipulations of $\alpha 2$ on Pavlovian conditioning (e.g. (Dixon et al., 2010). Given the heightened acute locomotor response to cocaine following ELS it is possible that the increase in conditioned activity simply reflects the increased salience of the unconditioned stimulus in ELS mice (i.e. the increased activity under cocaine).

Thus, in summary, our data suggest that ELS increases the acute locomotor stimulant effects of cocaine. This ability of ELS to enhance the acute effects of cocaine was more marked in $\alpha 2^{-/-}$ mice, consistent with animals without $\alpha 2$ receptors being more reactive to ELS, an effect that may reflect a stress-protective effect of $\alpha 2$-containing GABA$_A$ receptors. Overall, ELS also reduced sensitisation, an effect that was largely attributable to ELS abolishing sensitisation in

the heterozygous α2$^{+/-}$ mice, which had demonstrated a normal response to acute cocaine. Lastly, ELS enhanced conditioned activity independently of α2 genotype.

We interpret our findings in terms of ELS interactions with α2-containing GABA$_A$ receptors. However, ELS is known to have multiple long-term effects that might have contributed to our observations. In their characterisation of the ELS model employed here, Rice and colleagues (2008) report marked effects on hypothalamic-pituitary-adrenal (HPA) axis function during the stress procedure, some of which persist into adulthood. Thus, following ELS, steady-state corticotrophin releasing hormone (CRH) mRNA levels are reduced in adulthood, suggesting that the set-point of the HPA axis is reprogrammed during ELS, resulting in elevated basal plasma corticosterone. However, Gunn and colleagues previously reported facilitated glutamatergic innervation of the paraventricular CRF releasing neurons and increased expression of CRH at least at P20 following ELS (Gunn et al., 2013).

It is well known that prior stress exposure increases the stimulant effects of both psychostimulants and opioids, and that this effect is prevented by adrenalectomy (Deroche et al., 1992a, Deroche et al., 1992b, Deroche et al., 1993, Deroche et al., 1994) and reinstated by administration of corticosterone (Deroche et al., 1995). In keeping, pre-treatment with corticosterone facilitated cocaine sensitisation, though not the acute stimulant effect of cocaine, while adrenalectomy performed in adulthood completely blocked cocaine sensitisation (Prasad et al., 1998, Przegalinski et al., 2000). Repeated administration of corticosterone also induces sensitisation of the stimulant effects of another psychostimulant, amphetamine (Deroche et al., 1992a). Thus, it is plausible that persistent changes in corticosterone levels following ELS resulted in altered responses to cocaine in adulthood.

These phenomena have been suggested to reflect stress and corticosterone effects on dopaminergic transmission, as inhibiting corticosterone synthesis suppresses sensitisation of the increased dopamine response to cocaine in the accumbens (Rouge-Pont et al., 1995). In keeping, ELS has been reported to exert complex effects on various dopaminergic markers following cocaine exposure, with some evidence for increased cocaine-induced dopamine turnover following ELS (Gracia-Rubio et al., 2016). Possibly consistent with that observation, reduced binding of the dopamine transporter (DAT) ligand [3H]WIN 35,428 in accumbens and caudate-putamen of adult rats previously subjected to maternal separation has been

reported (Brake et al., 2004), suggesting impaired dopamine reuptake. In contrast, these authors found no differences between the ELS animals and their handled controls in [^{3}H]raclopride (D2-like receptors) or [^{3}H]SCH23390 (D1-like receptors) binding, suggesting no effects on dopamine receptor density.

However, the effects of corticosterone on dopaminergic transmission may not necessarily reflect changes in dopamine neurons themselves. One report (Ambroggi et al., 2009) indicates, instead, that there is a loss of glucocorticoid receptors from dopaminoceptive neurons (i.e., in striatum, medium spiny neurons (MSNs)), and in particular those neurons expressing dopamine D1-receptors. MSNs have a high density of α2-containing GABA$_A$ receptors (Dixon et al, 2010), and behavioural sensitisation can be induced by directly activating accumbal α2-containing GABA$_A$ receptors (Morris et al., 2008, Dixon et al., 2010). Since corticosterone administration is known to decrease expression of α2 subunits (Skorzewska et al., 2014), a plausible account of our findings might be that ELS acts to increase corticosterone levels, thereby decreasing α2 expression on D1-expressing medium spiny neurons.

While the loss of glucocorticoid receptors (Ambroggi et al., 2009) might suggest an influence on transcription via nuclear steroid receptors, an alternative mechanism might involve the conversion of corticosterone to neurosteroids acting at GABA$_A$-receptors (Brown et al., 2016). Blocking the enzyme 5α-reductase, which catalyses the main rate-limiting step in neurosteroid synthesis, in accumbens prevents the effects of dopaminergic drugs acting at D1 receptors (Frau et al., 2016). Thus, heightened levels of corticosterone might enhance the effects of dopaminergic agents such as cocaine by facilitating neurosteroid synthesis.

Although it is plausible that ELS achieves its effects on cocaine's actions by affecting expression of α2 subunits of GABA$_A$ receptors by chronically increasing levels of corticosterone, we cannot exclude alternative accounts. In the present experiments, ELS was applied in the mouse during early postnatal life, and it is unclear whether this particular timing is of significance. An augmentation of the locomotor response to cocaine has also been reported when the stressful procedure was carried out for 10 days, starting at postnatal day 25 (Lepsch et al., 2005), while adult exposure to stress also has profound influence on cocaine's effects, as outlined above. Thus, relating the present findings to the observation

that the association between GABRA2 haplotypes and the development of cocaine addiction was strongest in individuals who had suffered childhood trauma (Enoch et al., 2010) is difficult. As well as being unsure whether the effects we observe are restricted to imposition of stress during early postnatal days in the mouse, it should be considered that brain development in the mouse and human is rather different, with the mouse immediate postnatal period perhaps better related to human embryonic brain development in the third trimester of pregnancy. However, it is difficult to draw close parallels between particular aspects of development in mouse and human, and it is plausible that our mouse postnatal stress model encapsulates an earlier period in development of human neuronal systems employing α2-containing GABA$_A$ receptors.

Lastly, it is important to point out some limitations of our study. Firstly, we tested only male mice, as including females would have made the breeding and ELS procedure impossibly unwieldy. However, especially given reports that male and female mice may show different responses to early life stress (Kikusui et al., 2005), it would be important to carry out similar experiments in the future using female mice. Secondly, the ELS procedure resulted in significantly lowered body weights in the pups throughout development and at testing. However, these effects on body weight were small. Furthermore, whereas we saw reduced sensitisation following ELS, a period of undernutrition during the first postnatal weeks, which resulted in permanent changes in body composition and brain function, has been reported to enhance amphetamine sensitisation (Brioni et al., 1986). Most importantly, the current data set does not include direct measures of expression or function of α2-containing GABA$_A$ receptors. We have addressed this question in a previous publication (Mitchell et al., 2018).

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The authors declare they have no conflicts of interests.

Table and Figure Legends

Table 1: Make up of experimental groups. WT indicates mice with the genotype $\alpha 2^{+/+}$, HT: $\alpha 2^{+/-}$, KO: $\alpha 2^{-/-}$. n values represent the number of mice per condition

Figure 1: Left panel: Pups subjected to the ELS condition (S) were significantly lighter in body weight at p9 and p21 than control animals (NS). At p70, ELS mice weighed significantly less than their non-stressed counterparts. Whilst KO mice weighed less than WT and HT, this was independent of the ELS condition implemented.

Figure 2: Locomotor activity (metres travelled) during 2 days of habituation to the chamber and a single i.p. administration of saline (left panel) and following acute administration of cocaine (10 mg/kg) or its vehicle (right panel) in wildtype (WT), $\alpha 2^{-/-}$ (KO) and heterozygous (HT) mice. During habituation to the apparatus and injection procedure, all mice decreased their locomotor activity. However, KO mice showed an increase in locomotor behaviour in response to the sham saline injection on session 3 (session by genotype interaction: $F_{(4,302)}$ = 8.236, p < 0.001).

Acute cocaine response. On the first day of sensitisation, the locomotor response was higher in ELS compared to non-stressed animals (right panel; main effect of ELS: $F_{(1,145)}$ = 22.753, p < 0.001; ELS by drug interaction: $F_{(1,145)}$ = 23.510, p < 0.001). The increased locomotion was evident in response to cocaine administration but not in vehicle treated groups (main effect of ELS. Locomotor activation was also higher in KO ($\alpha 2^{-/-}$) animals compared to WT ($\alpha 2^{+/+}$)), but this effect was independent of drug treatment or ELS condition.

Figure 3. **A.** The effects of ELS and genotype on repeated locomotor activity differed according to drug administration (session by ELS by drug interaction: $F_{(9,1305)}$ = 3.778, p < 0.001; session by genotype by drug interaction: $F_{(9,1305)}$ = 3.008, p < 0.001). Repeated saline administration resulted in a significant habituation which did not differ according to either stress condition or genotype (day by ELS by genotype interaction: $F_{(18,657)}$ = 0.446, p = 0.977). Repeated cocaine administration resulted in a significant sensitisation, which was reduced by prior exposure to ELS (ELS by session interaction: $F_{(9,648)}$ = 3.605, p < 0.001; main effect of ELS: $F_{(1,72)}$ = 9.724, p < 0.01). The degree of sensitisation was altered according to genotype

(genotype by session interaction: $F_{(18,648)}$ = 3.374, p < 0.001), reflecting the previously reported absence of sensitisation in $\alpha2^{-/-}$ mice. However, any genotype effects observed were not altered by exposure to the ELS paradigm (ELS by genotype by session interaction: $F_{(18,648)}$ = 1.028, p = 0.425).

B: In order to test how exposure to ELS changed the degree of sensitisation, a measure of sensitisation was calculating by subtracting the acute response to cocaine on day 1 from the sensitised response on day 10. The degree of sensitisation observed was sensitive to manipulations of ELS and genotype (ELS by genotype by drug interaction: $F_{(2.157)}$ = 3.621, p < 0.05), which were present in cocaine treated groups (ELS by genotype interaction: $F_{(2.78)}$ = 3.310, p < 0.05) but not vehicle-treated animals (ELS by genotype interaction: $F_{(2.79)}$ = 0.317, p = 0.729). Exposure to ELS did not affect the degree of sensitisation in $\alpha2^{+/+}$ or $\alpha2^{-/-}$ mice. * ELS significantly reduced the expression of sensitisation in $\alpha2^{+/-}$ mice (p < 0.01).

WT: $\alpha2^{+/+}$; HT: $\alpha2^{+/-}$; KO: $\alpha2^{-/-}$

Figure 4: The presence of conditioned activity was confirmed following the sensitisation procedure in a single session in which all mice received an injection of saline vehicle. An increased locomotion in animals previously treated with cocaine was found when compared to those previously treated with vehicle. This effect was greater in stressed animals (main effect of ELS: $F_{(1,145)}$ = 5.981, p < 0.05; ELS by drug interaction: $F_{(1,145)}$ = 7.174, p < 0.05) and occurred to the same extent in all genotypes.

Table 1

Early-life	Genotype	Treatment	n
Control	WT	Saline	12
	HT		12
	KO		11
	WT	Cocaine	12
	HT		13
	KO		12
Stress	WT	Saline	12
	HT		20
	KO		12
	WT	Cocaine	14
	HT		15
	KO		12

Fig. 1
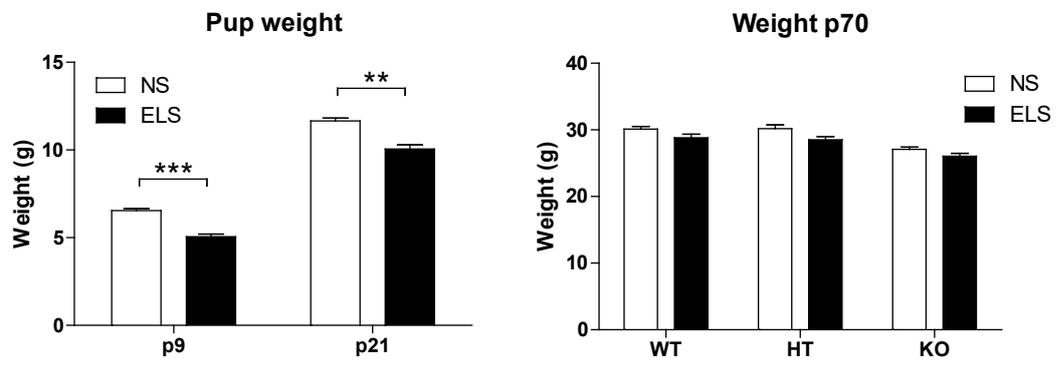
Pup weight

Weight p70

Fig. 2

A

Habituation

B   **Acute locomotor activation
Day 1 cocaine**

Fig. 3

A   **Behavioural Sensitisation**

Non-stress .... Early Life Stress

WT vehicle   HT vehicle   KO vehicle
WT cocaine   HT cocaine   KO cocaine

B **Expression of sensitisation**

WT veh   HT veh   KO veh
WT coc   HT coc   KO coc

22

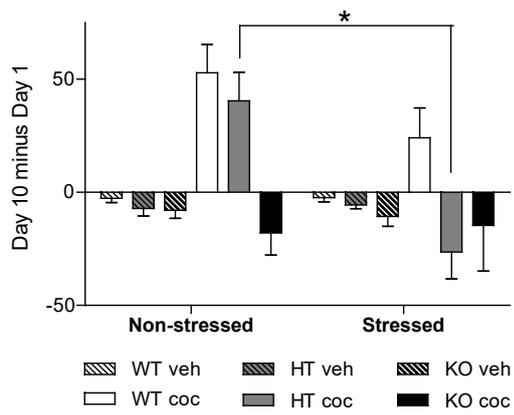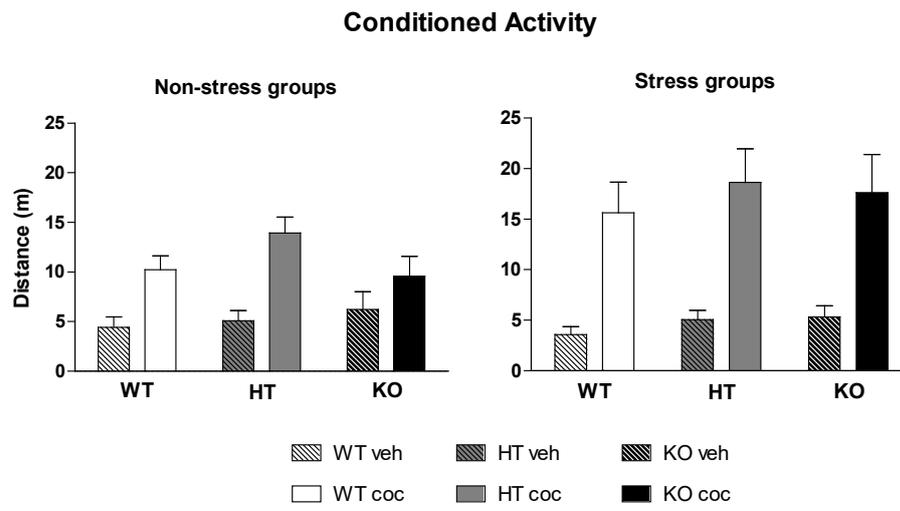Fig. 4

Conditioned Activity
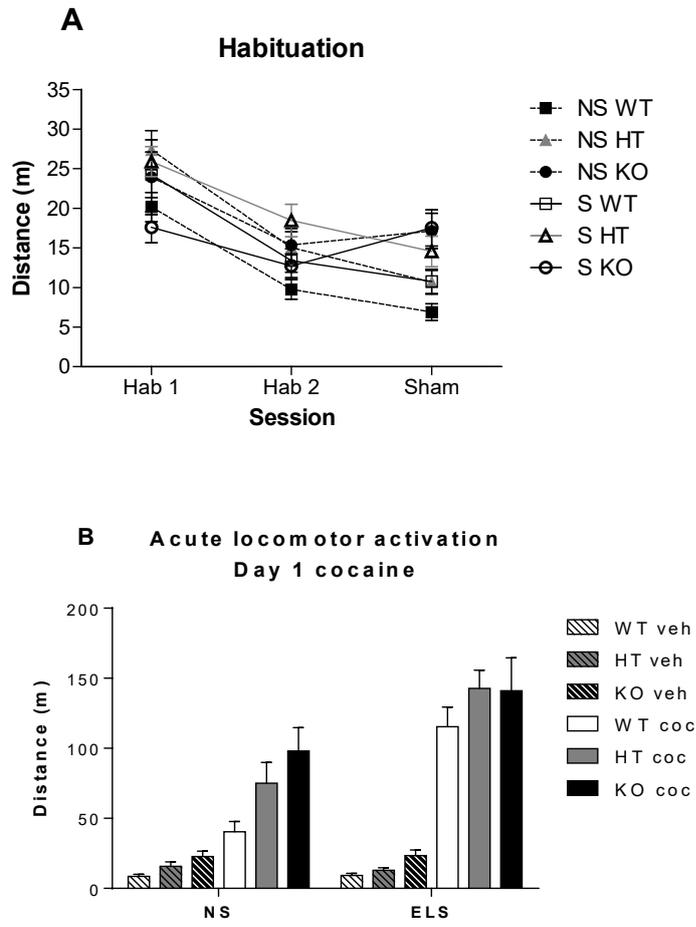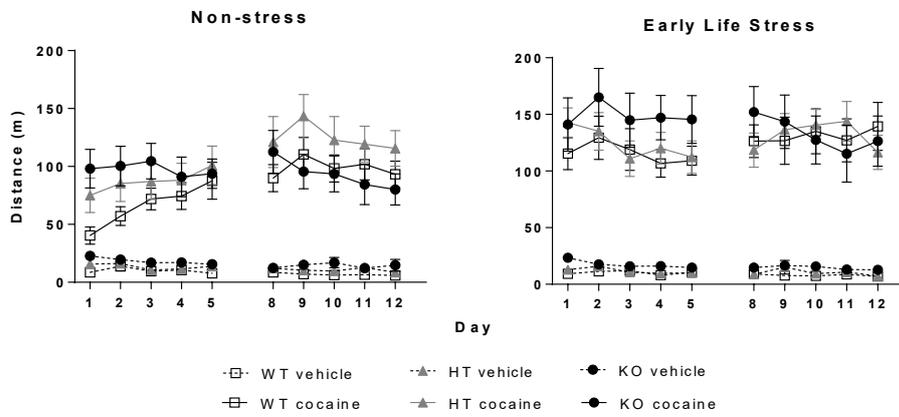
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