Socially induced behavioural plasticity precedes pronounced epigenetic differentiation in the CNS of Desert Locusts

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Abstract

Desert locusts show a dramatic form of socially induced phenotypic plasticity known as phase polyphenism. In the absence of conspecifics, locusts occur in a lone-living, shy and cryptic solitarious phase. Crowding with other locusts drives a rapid behavioural transformation towards gregarious behaviour that occurs over the span of few hours and is followed by changes in physiology, colouration and morphology to result in the full syndrome of the gregarious phase. We used methylation-sensitive amplified fragment length polymorphism (MS-AFLP) fingerprinting to compare the effect of social isolation, long-term crowding and acute crowding for one day on DNA methylation in the central nervous system. We find that rearing the offspring of chronically crowded locusts in social isolation leads to a pronounced differentiation of the neuromethylome within an individual's life-time. Crowding isolation-reared locusts for a day, however, has no significant effect on their MS-AFLP fingerprint. The pronounced differentiation of the neural methylome seen in long-term gregarious locusts is therefore unrelated to the acquisition and expression of gregarious behaviour, suggesting that it serves to consolidate long-term phase state.

Keywords: DNA methylation, phenotypic plasticity, phase change, *Schistocerca gregaria*

Introduction

Modification of DNA by cytosine methylation is emerging as an important mechanism in tailoring behavioural phenotypes to the prevailing environmental conditions, including the social environment (Szyf et al., 2008; Yan

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et al., 2014; Isles, 2015). The recent deluge of whole-genome methylation studies in both vertebrate and invertebrate species have uncovered correlations between environmentally induced differences in behaviour and differences in DNA methylation. Only in few instances, however, do we understand the role of DNA methylation in the chain of events from environmental signals to changes in the behavioural phenotype (Day et al., 2013; Isles, 2015). Do changes in the brain methylome effect behavioural change, or do they instead serve to consolidate changes that first arose through other mechanisms?

Phenotypic plasticity is particularly common in insects, a fact that has been implicated in their evolutionary success (Simpson et al., 2011). A striking example is provided by phase polyphenism in locusts. Locusts are grasshoppers (Acrididae) that can transform between two extreme phenotypes known as the solitarious and gregarious phase (Uvarov, 1966). The two phases differ profoundly in morphology and colouration, in endocrine, metabolic and reproductive physiology, and most importantly, in their behaviour (Pener and Simpson, 2009). The solitarious phase is cryptic and shy, and avoids other locusts; gregarious-phase locusts are active and mobile and seek out the presence of conspecifics, causing them to aggregate in swarms that can wreck agriculture on a continental scale. Several distantly related grasshopper species show phase polyphenism, with Migratory Locusts *(Locusta migratoria)* and Desert Locusts *(Schistocerca gregaria)* being amongst the most extreme and economically relevant. The sole direct environmental driver of phase change is the presence or absence of conspecifics. Solitarious Desert Locusts acquire gregarious behaviour within a few hours of forced crowding ((Roessingh and Simpson, 1994; Bouaïchi et al., 1995)). The converse process of behavioural solitarisation of long-term gregarious locusts is markedly slower, indicating that prolonged crowding causes a consolidation of the gregarious state. In Desert Locusts, phase state at hatching is additionally determined by transgenerational epigenetic inheritance (Islam et al., 1994). The parental effect on behavioural phase state is largely reversible within a life time, but the morphological phase extremes are only established after several successive generations of crowding or isolation.

Locust phase change provides a model for answering fundamental questions about the role of DNA methylation in socially induced behavioural plasticity. First, phase change can be fully controlled under laboratory conditions. Second, several lines of evidence indicate that locusts make ample use of DNA methylation despite having lost DNA (cytosine-5)-methyltransferase 3 (Robinson et al., 2011; Falckenhayn et al., 2013). (The claim of Wang et al. 2014 that *L. migratoria* has a DNMT3 orthologue is not supported by blast-searching the genome with insect DNMT3s as query sequences; Ott, unpublished observation). However, unlike flies (Glastad et al., 2014), locusts have retained orthologues of DNMT1, and their DNA is heavily methylated — on 1.6–1.9% of all genomic cytosines and on over 3% in

exons (in *S. gregaria;* Boerjan et al., 2011; Falckenhayn et al., 2013). These figures are more than ten times higher than in the honeybee, where DNMT3 is present and methylation is implicated in caste polyphenism (Lyko et al., 2010; Herb et al., 2012). A reduced representation bisulphite sequencing (RRBS) study in Migratory Locusts identified about 90 differentially methylated genes in the brains of solitarious and gregarious fourth-instar nymphs (Wang et al., 2014) but the gigantic size of the as yet unsequenced Desert Locust genome remains challenging (8,550 Mb, *vs.* 200 Mb for *Drosophila melanogaster*; Camacho et al., 2014).

DNA methylation may serve three not mutually exclusive roles in phase polyphenism: (1) it may be part of the effector cascade that *initiates* behavioural change; (2) it may underpin the *consolidation* of gregarious behaviour with prolonged crowding within a lifetime; or (3) it may mediate epigenetic *inheritance* of phase state across generations. To begin to distinguish between these possibilities, we have analysed methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) to compare the DNA methylation patterns in the CNS of Desert Locusts with identical parental histories, but different individual social histories.

Methods

Locust rearing

Desert Locusts (*Schistocerca gregaria* Forskål, 1775) were obtained from an inbred colony at the Department of Biology that had been maintained under crowded conditions for many generations. First-generation solitarious locusts were produced from this stock by isolating them within a day of hatching and rearing them in individual cages under visual, tactile and olfactory isolation from conspecifics (Roessingh et al., 1993). All locusts were maintained on an identical diet of fresh seedling wheat and dry wheat germ under a 12:12 photoperiod.

Experimental cohorts and treatments

All locusts were virgin adults sacrificed 17–21 days after the final moult. Long-term gregarious (LTG) locusts were removed from the gregarious colony as final larval instars, sexed, and set up as one all-male and one all-female cohort of 40 individuals each in separate tanks (length $\times$ width $\times$ height $= 40 \times 30 \times 25$ cm^3) in the controlled environment room that also housed the solitarious locusts. All solitarious locusts were sibling offspring from a single gregarious mother (first-generation solitarious, 1GS). A total of $N = 24$ locusts were split into three treatment groups of four males and four females each: (i) $n = 8$ 1GS locusts that never experienced crowding; (ii) $n = 8$ LTG locusts; and (iii) $n = 8$ behaviourally gregarised 1GS locusts. These

behaviourally gregarious locusts were produced by placing 1GS locusts in the tank that housed the 40 LTG virgins of the same sex (*i.e,* four 1GS males with the 40 LTG males; and four 1GS females with the 40 LTG females). Crowding lasted for the final 24 h before sacrifice, with unrestrained physical interaction between all locusts in the crowding tanks. Locusts were sacrificed by decapitation and immediate dissection under ice-cold saline. The brain (including the optic lobes, but not the retinae) and the chain of thoracic ganglia (prothoracic, mesothoracic and metathoracic ganglion) were dissected out and cleaned from contaminant tissue in ice-cold saline and snap-frozen on dry ice.

Methylation analysis by MS-AFLP

Differences in DNA methylation patterns were detected by MS-AFLP analysis in $n = 4$ independent samples per treatment group, for a total of $N = 12$ samples. Each sample comprised the pooled brains and thoracic ganglia from one arbitrarily chosen male and one arbitrarily chosen female within the same treatment group. DNA was extracted with the QIAamp DNA Micro Kit (QIAGEN) following the manufacturer's instructions.

The MS-AFLP protocol was based on Kronforst et al. (2008). Full experimental details including sequences of primers and PCR cycling parameters are given in the Supplementary Material. Briefly, for each sample of genomic DNA, one 500 ng aliquot was digested with EcoRI and MspI and another was digested with EcoRI and HpaII. The EcoRIMspI and EcoRI-HpaII restriction-digested products were ligated with EcoRI and HpaII-MspI adaptors. The first PCR (pre-amplification) used 1 µl of ligation product with 1 µl each of EcoRIpre and HpaII-MspIpre primers, and 7 µl of the reaction mix (0.8 µl 2.5 mM deoxynucleotide triphosphates (dNTPs), 1 µl 10× Paq5000 Hot Start Reaction Buffer, 0.3 µl Paq5000 Hot Start DNA Polymerase (500 units), 0.8 µl 25 mM $MgCl_2$, 4.1 µl sterile H_2O). Seven µl of these pre-amplified PCR products were diluted with 93 µl of H_2O and used as the template for selective amplification. Only one of twelve possible selective primer combinations was used in order to reduce the number of fragments visualised by gel electrophoresis to a manageable number. The selective PCR reaction mixture contained 1 µl pre-amplified product, 1 µl each of HpaII-MspI primer and EcoRI primer and 7 µl reaction mix (as above).

PCR products were diluted with 100 µl H_2O. Ten µl of diluted PCR product was mixed with 3 µl 1× loading buffer (Elchrom) and run on 9% poly(NAT) gels (Elchrom) on an Origins electrophoresis system (120 V, 81 minutes at 55°C). Gels were stained with SybrGold. Bands were scored as either present or absent. The resulting binary matrix was analysed for differentiation between groups by principal coordinates analysis (PCoA) and by analysis of molecular variance (AMOVA) in the R package MSAP (Pérez-Figueroa, 2013).

Results

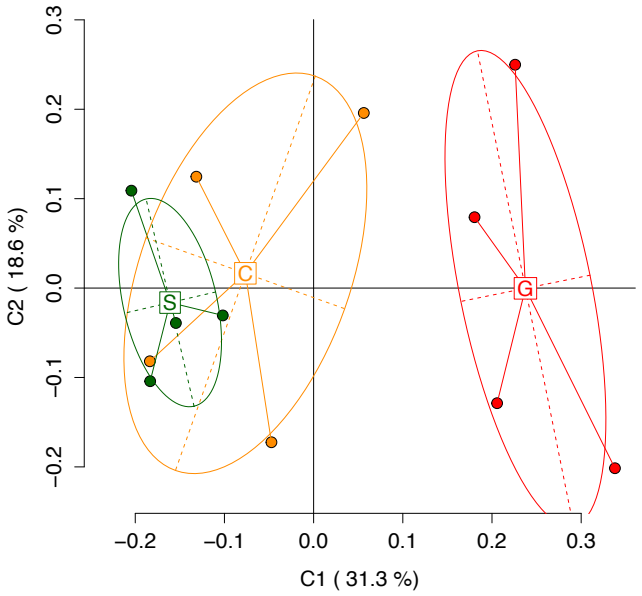

Figure 1. Principal Coordinate Analysis (PCoA) of epigenetic differentiation between uncrowded solitary-reared locusts (S), long-term gregarious locusts (G) and solitary-reared locusts crowded for 24 h (C), as identified by MS-AFLP. The first two coordinates (C1, C2) are shown with the percentage of variance explained by them. Group labels show the centroid for each group. Points correspond to individual MS-AFLP samples, ellipses represent their average dispersion around the group centroids.

We used MS-AFLP fingerprinting to compare the global pattern of DNA methylation (epigenetic differentiation) between (i) uncrowded locusts that had been reared in social isolation from hatching to adulthood; (ii) isolation-reared adult locusts that had been gregarised behaviourally by 24 h of crowding; and (iii) crowd-reared long-term gregarious locusts. Co-digestion with EcoRI and either one or the other of two methylation-sensitive restriction enzymes, HpaII and MspI, gives four possible combinations of bands (+/+, +/−, −/+, −/−). Their relation to the underlying methylation patterns are given in Table 1. We scored 102 unique bands (loci); out of these, 99 were identified as methylation-susceptible on the basis that they showed different digestion patterns with HpaII and MspI. Of these 99 methylation-susceptible loci, 53 showed different banding patterns between individual samples (*MS-polymorphic loci*).

The three treatment groups showed significant multi-locus differentiation in methylation pattern (AMOVA, $\phi_{ST} = 0.2086$, $p = 0.0114$). In

pair-wise comparisons, crowd-reared gregarious locusts showed significant differentiation both from uncrowded solitary-reared locusts ($\phi_{ST} = 0.3952$, $p = 0.0303$) and from solitary-reared locusts that had been crowded for 24 h ($\phi_{ST} = 0.2084$, $p = 0.0272$). In contrast, crowding for 24 h did not result in significant epigenetic differentiation in solitary-reared locusts ($\phi_{ST} = -0.1135$, $p = 0.9446$). These findings are visualised in Figure 1, where we have used principal coordinate analysis to generate a simplified representation of the multi-locus differences between the samples in a two-dimensional plane. The two axes represent the first two principal coordinates, which together explained 49.9% of the total variation. The gregarious samples are set well apart from both of the solitary-reared samples (uncrowded and 24 h crowded) by a shift along the first principal coordinate, indicating that the largest source of variance in multi-locus methylation is associated with long-term phase state.

The distinct methylation pattern of crowd-reared gregarious locusts, and the similarity between uncrowded and 24 h-crowded solitary-reared locusts, was also apparent in the proportions of fully methylated and unmethylated loci in the three treatment groups (Table 1). The proportion of unmethylated loci (HPA+/MSP+) was about twice as high in crowd-reared gregarious locusts (33%) than in either uncrowded solitary-reared locusts (14% of loci) or solitary-reared locusts after 24 h of crowding (17% of loci). Conversely, the proportion of fully methylated loci (HPA−/MSP−) was about twice as high in uncrowded and 24 h-crowded solitary-reared locusts (63% and 57% of loci, resp.) than in crowd-reared gregarious locusts (33% of loci).

Table 1. Proportion of methylation-sensitive restriction band patterns found in the CNS of locusts of different phase state, and their corresponding methylation status. [a]) + and − indicate the presence and absence, respectively, of a band following digestion with HpaII / MspI. [b]) may indicate methylation of either outer or both cytosines on one strand. [c]) HPA− / MSP− was taken to indicate hypermethylation rather than absence of target due to a genetic mutation (Pérez-Figueroa, 2013).

Banding pattern [a]	Methylation		solitary	24 h crowded	crowd-reared
HPA+ / MSP+	none:	5'-CCGG GGCC-5',	13.9%	16.7%	32.8%
HPA+ / MSP−	hemi: [b]	5'-CCGG GGCC-5',	10.1%	13.6%	15.7%
HPA− / MSP+	full internal:	5'-CCGG GGCC-5',	12.9%	12.6%	18.2%
HPA− / MSP−	hyper: [c]	5'-CCGG GGCC-5',	63.1%	57.1%	33.3%

Discussion

We have used MS-AFLP fingerprinting to compare the neural DNA methylation patterns of uncrowded solitary-reared and crowd-reared locusts with that of solitary-reared locusts after one day of crowding. We found evidence for a pronounced epigenetic differentiation between crowd-reared locusts and locusts reared in social isolation (Figure 1). These solitary-reared locusts were themselves the direct offspring of long-term gregarious parents. Our results therefore demonstrate that social isolation causes pronounced alterations in the neuromethylome within an individual's life-time. It would now be interesting to see whether isolation over multiple generations further deepens the epigenetic differences between the two phases.

The key finding of our study is that the CNS of locusts that have been crowded for 24 h does not show the differentiated methylome that is manifest in long-term gregarious locusts (Figure 1). One day of crowding is more than sufficient to establish gregarious behaviour (Roessingh and Simpson, 1994; Bouaïchi et al., 1995), yet the neural MS-AFLP fingerprint is still markedly different from that found in long-term gregarious locusts. In fact, crowding for 24 h caused no significant epigenetic differentiation. The MS-AFLP technique that we have used is insensitive to methylation at non-CpG sites (Bonasio et al., 2012), but it should nevertheless afford a representative fingerprint of the methylation landscape since 90% of methylated cytosines in the Desert Locust genome, and 97% within EST sequences, occur at CpG sites (Falckenhayn et al., 2013). It is also important to bear in mind that MS-AFLP resolves only a small random subset of CpG sites. Clearly, our results do not preclude that changes in methylation are already occurring at some sites within the first 24 h of crowding. The pronounced differentiation of the neuromethylome that is manifest in the fully established gregarious phase must, however, develop only some time after the first 24 h of crowding. It cannot, therefore, underpin the transition to gregarious behaviour, nor can it be required for its expression. Serotonin was previously found to be both necessary and sufficient to initiate behavioural gregarisation, by acting at least in part through activation of protein kinase A (Anstey et al., 2009; Ott et al., 2012). Our present results show that gregarious behaviour is dissociated from the manifestation of the differentiated methylome that we see in long-term gregarious locusts. Instead, our findings suggest a possible role for neural DNA methylation in the *consolidation* of gregarious behaviour. It is well established that although behavioural gregarisation is reversible within the life time of a locust, the speed and extent of solitarisation depend on the locust's age at the time of isolation and the length of time for which it has been crowded (Roessingh et al., 1993; Simpson et al., 1999). Long-term gregarious locusts solitarise only partially when isolated for four days as final instar nymphs; but when long-term solitarious locusts are re-isolated after 24–48 h of crowding, they return to fully solitarious

behaviour within 8 h. The resilience of gregarious behaviour to re-isolation thus increases with the length of time spent in crowded conditions. Our finding that 24 h of crowding does not induce the major epigenetic changes that manifest after prolonged crowding suggest that DNA methylation may provide a consolidation mechanism by which neurochemically mediated rapid changes in behaviour become more stable with time. Alternatively, or in addition, differences in neural DNA methylation may underpin long-term phase differences in the CNS that are not directly responsible for gregarious behaviour but represent adaptations to the respective life styles. An example are the profound differences in the size and structure of the brain (Ott and Rogers, 2010).

In ants, changes in exonic DNA methylation correlate with alternative splicing events such as exon skipping and alternative splice site selection (Bonasio et al., 2012). Wang et al. (2014) reported about 90 genes to be differentially methylated between long-term solitarious and long-term gregarious Migratory Locusts, and 45 genes to show phase-specific differential splicing. The causal relationship, however, between DNA methylation and alternative splicing has yet to be established in locusts. The work of Bonasio et al. (2012) on DNA methylation in ants found that some caste-specific methylation differences occur on orthologous genes in two species that belong to different subfamilies (*C. floridanus*, Formicinae; *H. saltator*, Ponerinae). It would be interesting to see whether orthologous phase-specific methylation differences similarly occur in *L. migratoria* and *S. gregaria*, which likewise belong to different subfamilies (Oedipodinae and Cyrtacanthacridinae, respectively.)

Of the methylation-sensitive loci covered in this study, a substantially greater proportion was unmethylated in crowd-reared locusts (Table 1), which may indicate that the consolidation of gregarious behaviour is associated with strong net demethylation. If so, pharmacological inhibition or RNAi knockdown of DNMT1 may accelerate the consolidation of the gregarious behavioural state. This would parallel findings in the honeybee, where depletion of DNMT3 by RNAi induces the development of queen-like phenotypes (Kucharski et al., 2008). Conversely, experimentally shifting the methylation balance in the CNS towards hypermethylation may provide a means for preventing or even reversing the consolidation of gregarious behaviour. At the group level, this would help the disbanding of locust aggregations, with possible implications for locust swarm management. In conclusion, our data demonstrate that phase change in the Desert Locust is associated with a major epigenetic shift in the neural methylome which manifests only after the initial transition from solitarious to gregarious behaviour.

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