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An ancestral mitochondrial DNA resembling a eubacterial genome in miniature

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Mitochondria, organelles specialized in energy conservation reactions in eukaryotic cells, have evolved from eubacteria-like endosymbionts[1–3] whose closest known relatives are the rickettsial group of α-proteobacteria[4,5]. Because characterized mitochondrial genomes vary markedly in structure[3], it has been impossible to infer from them the initial form of the proto-mitochondrial genome. This would require the identification of minimally derived mitochondrial DNAs that better reflect the ancestral state. Here we describe such a primitive mitochondrial genome, in the freshwater protozoon *Reclinomonas americana*[6]. This protist displays ultrastructural characteristics that ally it with the retortamonads[7,8], a protozoan group that lacks mitochondria[8,9]. *R. americana* mtDNA (69,034 base pairs) contains the largest collection of genes (97) so far identified in any mtDNA, including genes for 5S ribosomal RNA, the RNA component of RNase P, and at least 18 proteins not previously known to be encoded in mitochondria. Most surprising are four genes specifying a multisubunit, eubacterial-type RNA polymerase. Features of gene content together with eubacterial characteristics of genome organization and expression not found before in mitochondrial genomes indicate that *R. americana* mtDNA more closely resembles the ancestral proto-mitochondrial genome than any other mtDNA investigated to date.

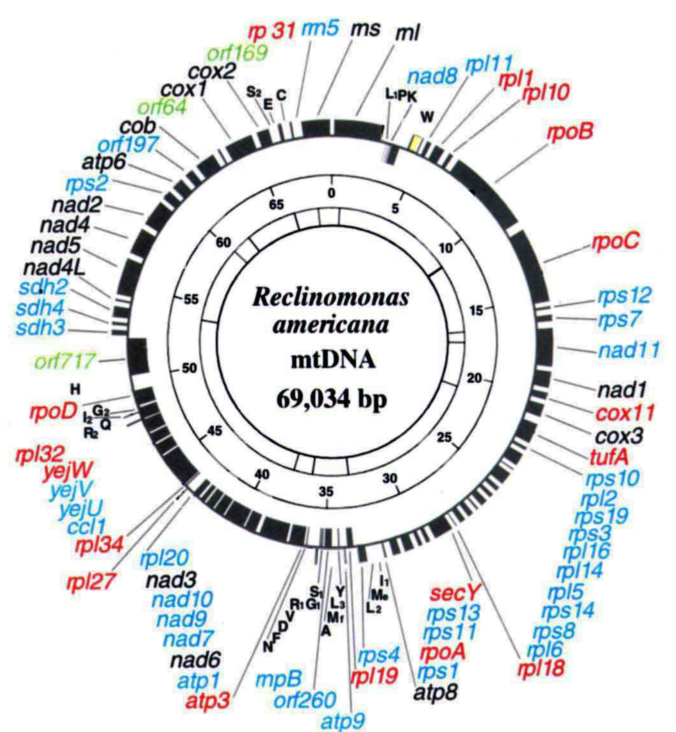

Figure 1 Gene map of the *Reclinomonas americana* mitochondrial genome, with the innermost circle showing the location of *Hin*dIII restriction sites. Identified protein-coding genes are listed in Table 1. The open reading frames (ORFs) *orf197* and *orf260* are homologous to *orf25* (*ymf39*) and *orf244* (*ymf16*), respectively, in liverwort (*Marchantia polymorpha*) mtDNA. Three other ORFs (*orf64*, *orf169* and *orf717*) are unique to *Reclinomonas* mtDNA. Other genes are *rns*, small subunit (SSU) rRNA; *rnl*, large subunit (LSU) rRNA; *rrn5*, 5S rRNA; *rnpB*, RNase P RNA. Transfer RNA genes are indicated by the one-letter amino-acid code, with subscripts denoting different genes specific for the same amino acid. Genes (represented by filled rectangles) shown on the outside of the outermost circle are transcribed in a clockwise direction, whereas those on the inside of the circle are transcribed anti-clockwise. Red, protein-coding genes unique to *R. americana* mtDNA; blue, protein-coding genes absent from vertebrate mtDNAs but generally or occasionally present in plant and protist mitochondrial genomes; green, unique ORFs. A single group II intron (yellow rectangle) is located in the *trnW* gene.

Currently, the inferred set of 'proto-mitochondrial genes' comprises 44 protein-coding genes that specify 23 components of complexes I–V of the electron transport chain, 18 mitoribosomal proteins, and 3 proteins involved in cytochrome c_1 biogenesis (Table 1). In addition, mtDNA encodes up to 3 ribosomal RNAs, up to 27 different transfer RNAs, and (rarely) the RNA subunit of mitochondrial RNase P. At present, therefore, a limited set of about 75 genes of assignable function can be traced directly to the proto-mitochondrial genome, by virtue of their presence in at least several, if not most, contemporary mtDNAs.

In order to provide a more comprehensive picture of mitochondrial genome organization and evolution within the unicellular eukaryotes, which make up the bulk of the biological diversity within the eukaryotic lineage, the Organelle Genome Megasequencing Program (OGMP) is systematically determining the complete mtDNA sequences of selected protists. One of the organisms chosen for this analysis is *Reclinomonas americana* (ATCC 50394), a recently described[6] heterotrophic flagellate. The 'jakobid' assemblage to which *R. americana* has been assigned shares specific

ultrastructural characteristics with the amitochondriate retortamonads (refs 7, 8 and C.J.O'K., M. A. Farmer and T. A. Nerad, manuscript in preparation), a group that is thought to have diverged from the main eukaryotic line before the acquisition of mitochondria[9]. Thus the jakobid flagellates may be ancestral, mitochondria-containing protists that represent an early offshoot of the eukaryotic lineage.

The AT-rich (74%) sequence of *R. americana* mtDNA (69,034 base pairs (bp)) assembles into a unique unicircular map, densely packed with genes that are distributed on both strands (Fig. 1). Intergenic spacer sequences make up only 8% of the mitochondrial genome, and only a single (group II) intron has been identified, within the *trnW* gene. There are a total of 92 genes to which a function can be assigned, including all of the 44 protein-coding genes previously found in one or more sequenced mtDNAs (Table 1), as well as 26 tRNA genes and 3 rRNA genes. Among the latter is a 5S rRNA gene[10] that until now had been found only in the mtDNAs of land plants[11] and the unicellular chlorophyte alga, *Prototheca wickerhamii*[12]. Also present are two unidentified open reading frames (ORFs) that are conserved in plant and some other protist mtDNAs, as well as three unique ORFs of >60 codons (see Fig. 1).

The 26 predicted tRNA species all have conventional secondary structures, with very few deviations from the canonical structure. They have the potential to read all codons used in *R. americana* mitochondrial protein-coding genes with the exception of ACN (threonine). The lone tryptophan tRNA gene (*trnW*) has a CCA anticodon, which is consistent with the use of the standard genetic code in *R. americana* mitochondria.

In addition to protein-coding and RNA structural genes already known from other mitochondrial genomes, *R. americana* mtDNA contains 18 genes of assignable function (identified by searches against public-domain sequence databases) that have not previously been reported in mtDNA (Table 1). These new mtDNA-encoded genes include one (*atp3*) that encodes an additional component of the electron transport chain, nine new mitoribosomal proteins (all large subunit), and a gene (*yejW*) that specifies a fourth component of a cytochrome c_1 biosynthesis pathway. Genes of a type never before found in mtDNA include ones encoding a translation factor (*tufA*), a secretory pathway protein (*secY*), a putative cytochrome oxidase assembly protein (*cox11*), and four components of a eubacterial-type ($\alpha_2\beta\beta'\sigma$) RNA polymerase (*rpoA-D*).

The presence of eubacteria-like *rpo* genes in *R. americana* mtDNA is especially intriguing, considering that the only mitochondrial RNA polymerase so far identified is a nucleus-encoded, single-polypeptide enzyme homologous to bacteriophage T3 and T7 RNA polymerases[13,14]. Given the evidence supporting a eubacterial, endosymbiotic origin of the mitochondrion[1–5], it is not obvious how a single-subunit, T3/T7-like RNA polymerase came to be present in this organelle, and why it is used in mitochondrial transcription instead of a multisubunit, eubacteria-like enzyme, homologous genes for which are encoded in all characterized

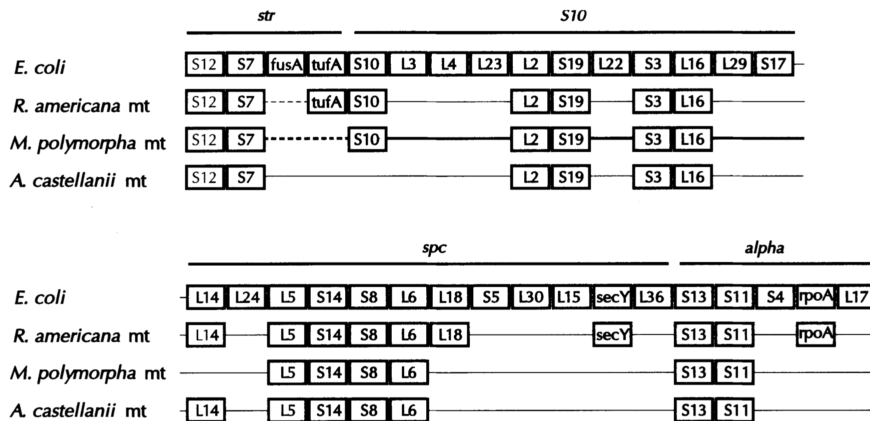

Figure 2 Conservation of ribosomal protein gene organization in the mitochondrial (mt) genomes of *Reclinomonas americana*, *Marchantia polymorpha* (liverwort) and *Acanthamoeba castellanii*, compared to the contiguous streptomycin (*str*), S10, spectinomycin (*spc*) and alpha operons of *E. coli*. Solid lines connect adjacent genes, dashed lines indicate the presence of additional genes that are not shown.

```
AAAACTATAAGAAATAGTTATTTAAAAAAAAGGAAAAAAAAATG....... tufA
TATGTGGGATTTCTTTGTGTGCTTAGAGAAAGGAATGTATATTTTATG... nad1
TTAATGTTTTTAATATTTTTGATATAGTAAAGGAAAATAAGATG....... nad11
TTAATTATTTAATATTTTTATTATTTTAAAAGGATTCTTATATG....... rps7
ATTCAAAGAAAATATTATAATAATTAATATGGGAATTTATTTTATG..... rpoB
ATAGAAGTGTTTGTATTTTTTAATATAAAAAGGTTGGTATAATATG..... nad4L
GTTTCAAAACTTATTTTTAAGTAAAATGAAAGGATATTTATATG....... cox2
TATAATTATAAATTCATAGAAAATAAAAAAAAGATAATCGCTTATG..... cox1
TATTGTATTTATAAATAATAAAAAAAACAAAGGAAATTCAAAATG...... cox3
                            ******
                         3' UUUCCUCAAGUAGGUCGA...SSU rRNA
```

Figure 3 Putative Shine–Dalgarno motifs (red) immediately upstream of inferred translation initiation codons (ATG, shown in blue) in representative *R. americana* protein-coding sequences. Potential base pairing between inferred Shine–Dalgarno sequences and the 3′-terminal region the *R. americana* mitochondrial SSU rRNA (green) is indicated by asterisks. The motif 5′-AAAGGA-3′ or one differing from it by a single nucleotide precedes at least 50 of the protein-coding sequences in *R. americana* mtDNA.

chloroplast genomes[15,16]. Although additional work will have to be carried out to verify that the *R. americana rpo* genes are functional, their sequences do not suggest that they are inactive pseudogenes.

Regardless of whether the *R. americana* mitochondrial *rpo* genes are actually functional, the simplest explanation for their presence is that they derive directly from the α-proteobacterial ancestor of mitochondria. If this is so, their absence from the mtDNA of most other eukaryotes implies that they were lost from the proto-mitochondrial genome at an early stage in mitochondrial evolution and were functionally replaced by a nuclear gene encoding a single polypeptide, T3/T7-like enzyme.

These observations prompt several questions. What was the evolutionary source of the T3/T7-like RNA polymerase now used as the mitochondrial RNA polymerase in many, if not most, eukaryotes? Is a multisubunit, eubacteria-like RNA polymerase used instead of (or in addition to) a T3/T7-like enzyme for mitochondrial transcription in some organisms, notably the jakobid flagellates? If so, might genes for such a eubacteria-like enzyme have moved into the nucleus in some cases? We presume that at some point in the evolution of mitochondria, recruitment of a nucleus-encoded, single polypeptide, T3/T7-like RNA polymerase allowed subsequent loss of the multisubunit, mtDNA-encoded activity, with eventual elimination of the corresponding genes from the mitochondrial genome. In this regard, it would be of interest to know whether T3/T7-like RNA polymerase sequences exist in *Reclinomonas* nuclear DNA (nDNA). A recent polymerase chain reaction (PCR) survey[17] provided evidence of such sequences in a number of unicellular eukaryotes, but not in *R. americana* or several other protists.

The *R. americana* mitochondrial genome encodes another typical eubacterial protein, a homologue of SecY, a component of the Sec protein-sorting machinery found in all eubacteria and plastids examined to date[18]. The presence of *secY* in *R. americana* mtDNA suggests that a Sec-based sorting pathway may act in the transport of proteins in *Reclinomonas* mitochondria. However, an intensive search of the completely sequenced yeast nuclear genome has failed to reveal any nDNA-encoded Sec homologues in *Saccharomyces cerevisiae*[19]. This situation may represent another instance of early loss from the proto-mitochondrial genome of genetic information for a eubacterial pathway, and its replacement by a new, nDNA-encoded one.

Vestiges of a prokaryotic operon organization are clearly evident in several gene clusters in *R. americana* mtDNA. These include the linkage groups *rpoB-rpoC*, *nad3-nad10-nad9-nad7*, *nad4L-nad5-nad4-nad2*, *sdh3-sdh4-sdh2*, and *yejW-yejV-yejU-ccl1* (=*yejR*). The most extensive bacteria-like linkage is found in a cluster of ribosomal protein genes that corresponds to the contiguous *str*, S10, *spc* and α operons of *Escherichia coli* (Fig. 2). By comparison with

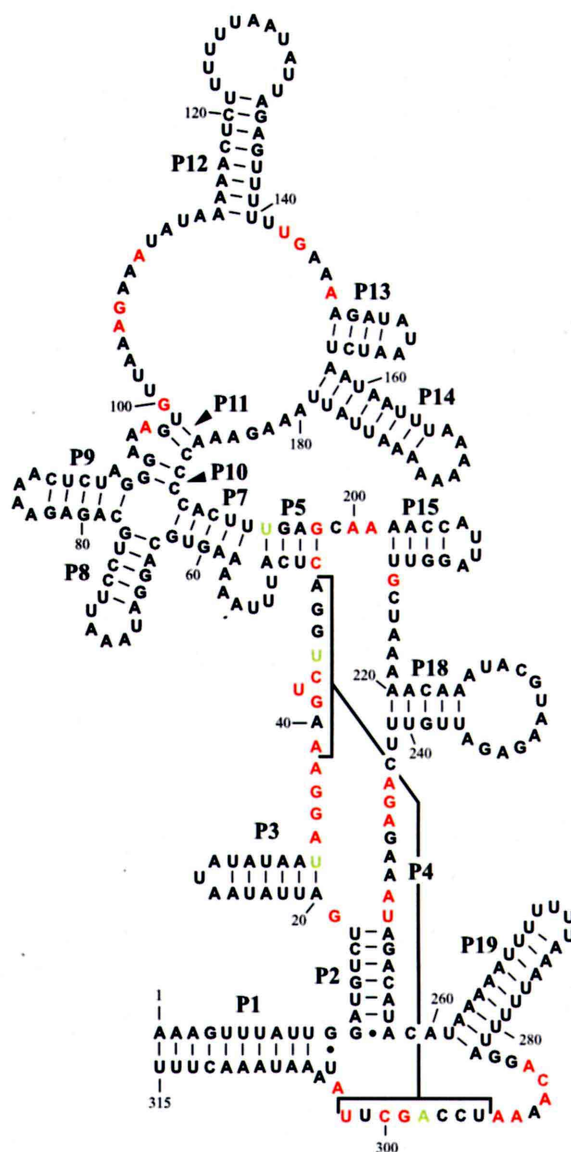

Figure 4 Secondary structure of *R. americana* RNase P RNA. The model is based on the *E. coli* secondary structure, and helical regions are numbered accordingly[28]. Residues in red match the bacterial consensus model[28], whereas those in green do not. A long-range tertiary interaction (P4) is supported by compensating base changes at two positions, relative to the *E. coli* model. (Figure courtesy of J. R. Brown, Indiana University).

Table 1 Protein-coding genes of identified function in *Reclinomonas americana* mitochondrial DNA

Electron transport and ATP synthesis	
Complex I	*nad 1, 2, 3, 4, 4L, 5, 6, 7, 8, 9, 10, 11*
Complex II	*sdh 2, 3, 4*
Complex III	*cob*
Complex IV	*cox 1, 2, 3*
Complex V	*atp 1, 6, 8, 9,* ***3***
Translation	
Small subunit ribosomal proteins	*rps 1, 2, 3, 4, 7, 8, 10, 11, 12, 13, 14, 19*
Large subunit ribosomal proteins	*rpl 2, 5, 6, 11, 14, 16* ***1, 10, 18, 19, 20, 27, 31, 32, 34***
Elongation factor	***tufA***
Transcription	
Core RNA polymerase	***rpo A, B, C***
Sigma-like factor	***rpoD***
Protein import/maturation	
Cytochrome oxidase assembly protein	***cox11***
SecY-type transporter	***secY***
ABC transporter	*yej R(=cc11), U, V,* ***W***

Genes in bold and underlined are unique to *R. americana* mtDNA; the remainder have previously been found in mtDNA in other eukaryotes. An additional five protein-coding genes of unknown function are present in *R. americana* mtDNA (see Fig. 1).

clusters containing the same genes in the mitochondrial genomes of *Marchantia polymorpha*[20] and *Acanthamoeba castellanii*[21], it can be seen that the *Reclinomonas* cluster retains genes that have been deleted from one or both of the other two mtDNAs. These retained genes include *rpl18* as well as the non-ribosomal protein genes *tufA*, *secY* and *rpoA*. But it is striking that the three mtDNAs share specific deletions compared to the corresponding *E. coli* operons, including *rpl3-rpl4-rpl23* (between *rps10* and *rpl2*), *rpl22* (between *rps19* and *rps3*), *rpl29-rps17* (between *rpl16* and *rpl14*), *rpl24* (between *rpl14* and *rpl5*) and *rps5-rpl30-rpl15* (downstream of *rpl6*). Other specifically mitochondrial linkage groups are shared among these three eukaryotes; these clusters include *nad4-nad2-rps2* (in both *R. americana* and *A. castellanii* mtDNAs) as well as *sdh4-*(*sdh2*)*-nad4L* (ref. 22). Taken together, these comparisons suggest that various mitochondrion-specific features of gene organization had already been established in the mitochondrial genome of the most recent common ancestor shared by *Reclinomonas* and other eukaryotes. This conclusion strongly reinforces the concept of a single origin of the mitochondrial genome[5,23], arguing against the idea that *R. americana* might have acquired its relatively conserved mitochondrial genome through a different, more recent endosymbiotic event.

As a further indication of eubacterial character, we note that a Shine–Dalgarno-type interaction[24] is theoretically possible in the mitochondrial translation system of *Reclinomonas*, whereas this is not the case in other eukaryotes. In the *Reclinomonas* mitochondrial small subunit (SSU) rRNA, a pyrimidine-rich sequence occurs at the same position as the anti-Shine–Dalgarno sequence in *E. coli* 16S rRNA (the inferred 3′-end of the *Reclinomonas* SSU rRNA is 5′-CUCCUUU$_{OH}$, compared to 5′-CUCCUUA$_{OH}$ in *E. coli* 16S rRNA). A complementary purine-rich sequence (often 5′-AAAGGA-3′) is located between 2 and 12 nucleotides upstream of the inferred initiation codon of most protein-coding genes in *Reclinomonas* mtDNA (Fig. 3).

Finally, a gene encoding an RNase P RNA is present in *R. americana* mtDNA. Such a gene has previously been identified only in the mtDNA of *S. cerevisiae* and a few other fungi[25], including most recently *Aspergillus nidulans*[26]. In contrast to the very AU-rich fungal mitochondrial RNase P RNAs[27], the inferred *R. americana* homologue is strikingly eubacterial, displaying almost all of the evolutionarily conserved primary sequence and secondary structural motifs of a phylogenetic-minimum bacterial consensus RNase P RNA[28] (Fig. 4). At an estimated size of 311 nucleotides (nt), the *R. americana* RNase P RNA is only slightly larger than its smallest known eubacterial homologue, that from *Mycoplasma fermentans* (276 nt)[28]. Like the *Mycoplasma* RNase P RNA, the *Reclinomonas* one has an extra helix (P19) near the 3′-end and lacks helices P16 and P17. However, unlike the *Mycoplasma* homologue, the *Reclinomonas* RNase P RNA retains helices P12, P13 and P14 of the *E. coli* secondary structure (Fig. 4).

The 69-kbp *R. americana* mtDNA encodes a total of 67 protein-coding genes (including two unidentified but conserved ORFs and three unique ORFs), compared with 470 protein-coding regions in the 580-kbp genome of the eubacterium, *Mycoplasma genitalium*[29], and 1,743 protein genes in the 1.8-kbp *Haemophilus influenzae* genome[30]. Thus protein-coding gene density is similar in the three genomes (1 gene per 1.0–1.2 kbp). Comparison of the *Mycoplasma* and *Haemophilus* genomes suggested that their different gene contents reflect "profound differences in physiology and metabolic capacity between these two organisms"[29], the differential reduction and tailoring of genetic information presumably being driven by the particular biological niches occupied by these two bacteria. In this context, the *Reclinomonas* mitochondrial genome may be viewed as an extreme example of eubacterial genome reduction, such that the only genes remaining are related to mitochondrial gene expression (transcription, RNA processing and translation) and biogenesis of the protein complexes required for electron transport and coupled oxidative phosphorylation (including components implicated in mitochondrial protein transport and haem biosynthesis).

In summary, *R. americana* mtDNA provides a striking example of a minimally derived mitochondrial genome, one that offers new insights into gene content, organization and expression in the ancestral proto-mitochondrial genome. *R. americana* mtDNA contains the largest gene set uncovered to date, with many new genes, including some for new mitochondrial functions. Of particular note is the first documented presence of *rpo* and *sec* genes in mtDNA, a finding that has implications for our views about the origin and evolution of the mitochondrial transcriptional apparatus and protein import machinery. *R. americana* mtDNA displays more pronounced eubacterial features of gene organization (linkage) and gene expression (Shine–Dalgarno potential) than any other sequenced mtDNA, further testifying to the eubacterial ancestry of the mitochondrial genome. Continued exploration of mitochondrial genomes within the most ancestral unicellular eukaryotes can be expected to refine further our understanding of the nature of the most recent common ancestor of extant mitochondrial genomes and the divergent pathways mitochondrial genome evolution has since taken in different eukaryotic lines. □

Methods

The sequence of the *R. americana* mitochondrial genome has been determined under the auspices of the Organelle Genome Megasequencing Program (OGMP). Details of growth conditions for *R. americana* and isolation and cloning of its mtDNA will be presented in conjunction with a more detailed description and analysis of the complete sequence (G.B. *et al.*, unpublished results). DNA sequencing, data entry and sequence analysis were performed as described[21].

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Behavioural stress facilitates the induction of long-term depression in the hippocampus

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The induction of activity-dependent persistent increases in synaptic efficacy, such as long-term potentiation (LTP), is inhibited by behavioural stress[1,2]. The question arises whether stress also affects the ability to induce persistent decreases in synaptic efficacy, such as long-term depression (LTD)[3–5]. We now report that the induction of stable homosynaptic LTD in the CA1 area of the hippocampus of awake adult rats is facilitated, rather than inhibited, by exposure to mild naturalistic stress. The same stress blocked the induction of LTP. The effects of such stress were short lasting: acclimatization to, or removal from, the conditions that facilitated LTD induction led to a rapid loss of the ability to elicit this form of plasticity. The time window in which LTD could be reliably elicited was prolonged by inducing anaesthesia immediately after the stress. These data reveal that even brief exposure to mild stress can produce a striking shift in the susceptibility to synaptic plasticity in the awake animal.

The conditions necessary to induce LTD in freely moving awake animals have proved elusive[6–8]. As exposure to inescapable aversive stimuli has been reported to reduce high-frequency stimulation (HFS)-induced LTP in the hippocampus[1,2], we wondered whether stress would also affect the ability to elicit LTD. To investigate this, three experimental protocols that produce mild naturalistic stress were compared with three related but non-stressful protocols.

The first set of experiments compared the ability of low-frequency stimulation (LFS) to induce LTD in recording-acclimatized (non-stressed) and recording-naive (stressed) rats. Animals that had been acclimatized extensively to the brightly lit recording box and recording procedure after electrode implantation showed no signs of behavioural stress and had low serum levels of corticosterone. In contrast, rats that had not been handled during the 2-week period after surgery showed behavioural signs of stress, including behavioural 'freezing' (remaining in a fixed immobile position), defecation and urination for up to 10 min on first being placed in the brightly lit unfamiliar recording box. They also had raised serum corticosterone (see Methods). Consistent with previous studies[6–8] in acclimatized animals, LFS (900 pulses at 3 Hz) of the ipsilateral Schaffer collateral/commissural fibres in the stratum radiatum of the CA1 region did not affect the amplitude of the field excitatory postsynaptic potential (EPSP) (Fig. 1a), although LTP was reliably induced by high-frequency stimulation (200 Hz; Fig. 1b). In contrast, reliable LTD was induced in stressed animals. Thus, when the LFS protocol was applied to unhandled, recording-naive animals 40 min after they had been placed for the first time in the recording box, stable homosynaptic LTD was elicited (Fig. 1c; see also Fig. 3c). Consistent with previous reports on the effects of stress[1,2], LTP induction was blocked in recording-naive rats (Fig. 1d). Significantly, the stress of transferring recording-naive animals from the home cage to the new environment did not produce a baseline change in synaptic transmission at the time of recording that might have affected the ability to induce LTD (Fig. 1e).

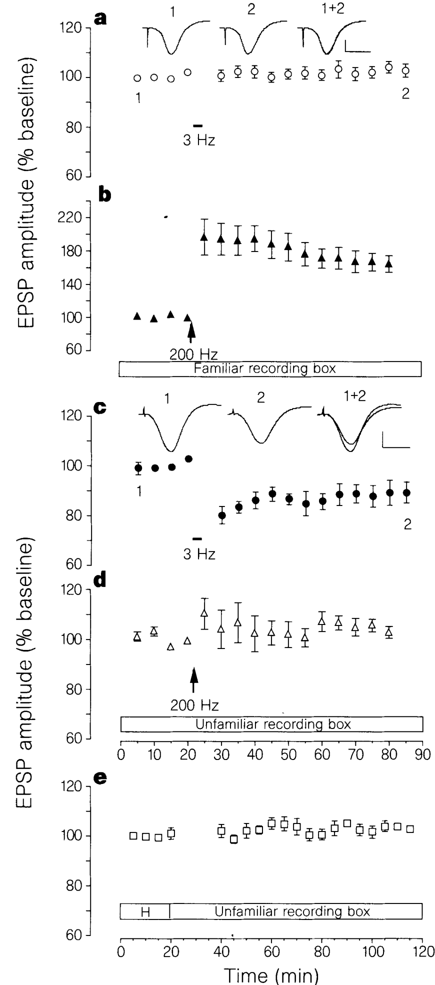

Figure 1 Novelty stress enables low-frequency stimulation (LFS) to induce LTD and prevents high-frequency stimulation (HFS) eliciting LTP in freely moving, recording-naive rats. **a**, LFS (3 Hz; bar) failed to induce LTD of the field EPSP amplitude in rats that had been acclimatized to the recording box and procedure ($n = 10$; 101.8 ± 2.4% of baseline 30 min after LFS). Inset: field potentials (average of 10 consecutive sweeps) from one experiment at the times indicated by the numbers. Horizontal bar: 10 ms; vertical bar: 1 mV. **b**, HFS (200 Hz, arrow) induced reliable LTP in acclimatized animals ($n = 9$; 163.4 ± 9.9% of baseline at 60 min; $P < 0.01$ compared with pre-HFS baseline). **c**, Stable LTD was induced by LFS in unhandled, recording-naive animals when placed for the first time in a brightly lit recording box ($n = 7$; 84.8 ± 4.9% of baseline at 30 min and 89.2 ± 4.2% 60 min after LFS; $P < 0.01$). Inset: field potentials from one experiment. **d**, HFS failed to induce potentiation of the EPSP in unhandled, recording-naive animals when placed for the first time in the brightly lit recording box ($n = 4$; 102.6 ± 2.2% of baseline at 60 min after the tetanus). **e**, There was no baseline change between recording in the home cage (H) and the unfamiliar recording box in recording-naive animals ($n = 8$; $P > 0.05$).