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Repeated linalyl acetate inhalation attenuates the stress-evoked immediate-early gene programme in the mouse hippocampus and olfactory bulb: a reanalysis of public transcriptomic data

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Research Article

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Linalyl acetate and the stress-evoked activation programme

Title page

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Running title: Linalyl acetate and stress-evoked brain activation

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Abstract

Background: Insomnia is associated with a state of physiological hyperarousal rather than with a simple loss of sleep drive. Aromatherapy is used as self-care for this kind of sleep problem, but its pharmacological basis is unclear. Linalyl acetate (LA) is a major constituent of bergamot and lavender oils. A recent study deposited brain transcriptomes from mice that inhaled LA and were sampled immediately after an acute stressor, and interpreted a module of immediate-early genes (IEGs) as evidence of neurotrophin-driven activation, without reporting the direction of change.

Methods: We reanalysed the deposited hippocampal and olfactory bulb microarrays (GSE316912, GSE316919; vehicle, 1% and 10% LA, n = 3 per group; tissue taken immediately after the final tail suspension test) with a pre-specified 24-gene IEG set. Significance was assessed with moderated t-statistics, competitive rank tests and exhaustive sample-label permutation (84 labellings). The LA profile was compared with public datasets for acute stress (GSE100236), sleep deprivation (GSE33302), chronic mild stress (GSE151807), chronic diazepam (GSE76700), chronic fluoxetine (GSE118669, GSE43261) and linalool inhalation in rats (GSE19589, GSE90923).

Results: Acute handling, restraint and cold swim each increased all 24 IEGs in the hippocampus, and sleep deprivation increased them. After LA inhalation the same genes were decreased: 21 of 22 detected IEGs in the hippocampus and 22 of 23 in the olfactory

bulb (label-permutation P = 0.012 and 0.024). LA therefore moved the stress-evoked activation programme in the direction opposite to the acute stressors that induce it. Four weeks of chronic mild stress, by contrast, also lowered these genes in all four brain regions examined, so the direction of the IEG programme depends on the duration of the stressor and does not by itself distinguish relief from chronic stress. Chronic diazepam produced the same direction in the hippocampus (20/23 decreased) and amygdala (19/23); fluoxetine did not (14/24 and 0/24). Diazepam-regulated genes outside the IEG set were not reproduced by LA (P = 0.083 and 0.82). In exploratory analyses, hippocampal *Crh* mRNA was lower after LA (P = 0.012) and olfactory bulb *Oxtr* and *Fkbp5* mRNA were higher (P = 0.024 and 0.012). In rat hypothalamus, linalool gave inconsistent IEG effects across two datasets and did not reduce glucocorticoid-responsive genes.

Conclusions: Repeated LA inhalation is associated with attenuation of the acute stress-evoked activation programme in the hippocampus and olfactory bulb, in the direction opposite to arousal and acute stress but the same as chronic stress. This reverses the published interpretation and supports a framework in which inhaled bergamot constituents act on the stress that sustains arousal rather than by direct sedation. It yields testable predictions for c-Fos mapping, oxytocin signalling and flumazenil antagonism in aroma studies of stress-related sleep disturbance.

Introduction

Insomnia is associated with physiological hyperarousal rather than with a simple loss of sleep drive. People with chronic insomnia secrete more ACTH over 24 h than matched controls (4.2 ± 0.3 vs 3.3 ± 0.3, P = 0.04), with more secretory pulses and an unchanged circadian pattern [9], and show higher high-beta EEG power and higher resting heart rate during the day [10]. These studies are cross-sectional and do not establish whether the arousal precedes the insomnia or follows it. Hypnotic drugs act on the arousal rather than on what drives it: benzodiazepine receptor agonists potentiate GABA_A receptors and orexin receptor antagonists block orexin signalling, and neither addresses an upstream stressor. This has motivated interest in interventions aimed at stress itself, although no trial has compared the two targets directly.

Aromatherapy is used widely as self-care for this purpose. Essential oils from bergamot (*Citrus bergamia*) and lavender (*Lavandula angustifolia*) are the most common choices, and their main shared constituents are linalyl acetate (LA) and linalool. In a Calabrian bergamot oil, limonene accounted for 41.8%, LA for 26.7% and linalool for 7.0% [1]. In lavender oil, the ISO 3515 profile specifies 25–47% LA, 20–45% linalool and at most 1% limonene [2]. LA and linalool are therefore the candidate active constituents common to both oils.

Animal work supports an action on stress rather than on sleep circuits alone. Bergamot oil at 2.5% attenuated the corticosterone response to the elevated plus maze in rats [3]. Inhaled linalool prolonged pentobarbital-induced sleep in mice [4]. Linalool odour produced anxiolytic effects that were absent in anosmic mice and abolished by flumazenil, implicating benzodiazepine-sensitive GABA_A receptors [5]. None of these studies measured what the brain's transcriptional output does when an odorant and a stressor are applied together.

Immediate-early genes (IEGs) provide that readout. *Fos*, *Arc*, *Egr1* and *Nr4a1* are transcribed within minutes of neuronal activation. Their mRNA rises with wakefulness and with acute stress and falls when activity is reduced, which makes the IEG programme a direct index of stress-evoked brain activation. Ferdousi et al. recently deposited hippocampal and olfactory bulb transcriptomes from mice given LA by inhalation for 7 days and sampled immediately after a tail suspension test [6]. They identified an IEG-containing network module and presented it as evidence of neurotrophin-receptor activation, but did not report the direction of change. Because every animal in that study met an acute stressor just before sampling, the dataset is in effect a test of whether LA alters the brain's transcriptional response to stress.

We asked three questions with public data only. First, in which direction does LA move the stress-evoked IEG programme? Second, how does that direction compare with the stressors and with arousal that induce the same genes, and with drugs that act through GABAergic and serotonergic mechanisms? Third, is the effect reproduced by linalool in independent rat datasets in which a stressor was also applied?

Methods

Datasets

All data were obtained from the NCBI Gene Expression Omnibus (GEO) on 5 November 2026. No new animal experiments were performed. Table 1 lists the datasets.

Table 1. Public datasets analysed

GEO	Species, region	Contrast (n per group)	Platform	Values used
GSE316912	Mouse hippocampus	Vehicle (3) vs 1% LA (3) vs 10% LA (3); 30 min/day inhalation for 7 d, TST daily	Clariom S	Authors' SST-RMA log2 signals (supplementary xlsx)

GEO	Species, region	Contrast (n per group)	Platform	Values used
GSE316919	Mouse olfactory bulb	As above (same study)	Clariom S	As above
GSE76700	Mouse hippocampus, amygdala, cortex	Vehicle (3) vs diazepam i.p. twice daily for 10 d (3)	MTA 1.0	Series matrix, gene level (SST-RMA)
GSE118669	Mouse dentate gyrus	Vehicle (8) vs fluoxetine 15 mg/kg/d for 3 wk (8)	Mouse 430 2.0	Series matrix (RMA)
GSE43261	Mouse dorsal and ventral dentate gyrus	Untreated (8) vs fluoxetine responders (7)	Mouse 430 2.0	Series matrix
GSE33302	Mouse hippocampus	Undisturbed (9) vs 5 h sleep deprivation (8)	Mouse 430 2.0	Series matrix (RMA)
GSE100236	Mouse whole hippocampus	Home cage (5) vs handling/novelty, restraint or cold swim, 45 min after onset (5 each)	RNA-seq	Authors′ edgeR results
GSE151807	Mouse hippocampus, amygdala, prefrontal cortex, cerebral cortex	Control (3) vs 4 wk chronic mild stress (3), per region	Mouse 430 2.0	Series matrix (GC-RMA, median-centred per gene)
GSE19589	Rat hypothalamus	Control, 2 h restraint, (R)-(−)-linalool, linalool + restraint (4 each)	Rat 230 2.0	Series matrix (MAS5)
GSE90923	Rat hypothalamus	Control, 2 h restraint, (R,S)-linalool + restraint (6 each)	Rat 230 2.0	Series matrix (FARMS)

LA, linalyl acetate; TST, tail suspension test. In the LA study, tissue was collected immediately after the final TST on day 7 [6].

Preprocessing

Probes or transcript clusters were mapped to gene symbols using the GEO platform annotation. Probes mapping to several symbols were removed. For each symbol, the probe with the highest mean signal was kept; for the LA files, duplicate symbols were averaged. Lowly expressed genes were removed (mean log2 signal ⩽ 5 for Clariom S and MTA; lowest quartile for Mouse 430 2.0 and GSE90923; MAS5 signal < 100 for GSE19589 after log2 transformation with a floor of 16). In the olfactory bulb file, 27 cells had been converted to dates by spreadsheet software, and the affected rows were removed. The GSE151807 matrix is median-centred per gene across all 24 samples, so absolute expression is not recoverable and an intensity filter cannot be applied within that dataset; expressed probes were instead defined from GSE33302, which uses the same platform and reports absolute RMA values. Per-gene centring is a constant shift and does not affect within-region contrasts. Raw CEL files could not be renormalised because the Rat 230 2.0 chip definition file was not obtainable in our environment. Every rat analysis therefore used within-dataset statistics only.

Pre-specified gene sets

The IEG set comprised 24 rapid activity-regulated genes, defined before the analyses: *Fos, Fosb, Junb, Egr1, Egr2, Egr3, Egr4, Arc, Npas4, Nr4a1, Nr4a2, Nr4a3, Ier2, Dusp1, Btg2, Cyr61, Atf3, Gadd45b, Gadd45g, Sik1, Klf10, Dusp6, Ier5* and *Klf4*. The glucocorticoid-responsive set comprised 10 genes: *Sgk1, Fkbp5, Tsc22d3, Errfi1, Klf9, Per1, Cdkn1a, Ddit4, Klf15* and *Nfkbia*.

Statistics

For each contrast, gene-wise moderated t-statistics were computed with empirical Bayes variance shrinkage [7], implemented in Python. The primary LA contrast pooled both doses against vehicle (6 vs 3); each dose is also reported. Benjamini–Hochberg FDR was applied genome-wide. For GSE100236, signed z-scores were derived from the authors' P values.

Gene-set effects were assessed in two ways. The competitive test compared the statistics of set genes with all other genes (two-sided Mann–Whitney U). Genes are correlated, so this test can overstate significance. The self-contained test therefore permuted sample labels. The set score was the mean t of set genes minus the mean t of all genes. Every distinct relabelling was evaluated: 83 alternatives for the LA datasets (3 vs 6), 69 for GSE19589 (4 vs 4) and 923 for GSE90923 (6 vs 6). One-sided $P = (k + 1)/(N + 1)$, where k is the number of relabellings at least as extreme as the observed score. With 83 relabellings the smallest attainable P is 0.012.

To test resemblance beyond IEGs, we took genes with FDR < 0.05 in the diazepam hippocampal contrast, excluding IEGs. The score was the mean LA t of diazepam-increased genes minus that of diazepam-decreased genes, assessed by the same label permutation. One-sided P values from the two rat datasets were combined with Fisher's method.

After the pre-specified analyses, we examined GABA_A receptor subunit genes and 20 stress-axis and neuropeptide genes in exploratory analyses. These results are labelled as exploratory and were not used to support the main conclusions.

Analyses used Python 3.13 with pandas, NumPy, SciPy and statsmodels. Scripts and the list of accessions are archived at Zenodo, doi:10.5281/zenodo.24119697.

Results

LA inhalation lowers the stress-evoked IEG programme in the hippocampus and olfactory bulb

All animals in the LA study met an acute stressor, the tail suspension test, immediately before tissue collection [6]. The IEG measurements below therefore report the transcriptional response to that stressor in vehicle- and LA-exposed mice.

In the hippocampus, 21 of 22 detected IEGs were lower after LA than after vehicle (median log2 fold change −0.63; competitive $P = 9.8 \times 10^{-12}$; Table 2, Fig. 1). *Arc* was the only gene genome-wide with FDR < 0.05 (log2FC −1.54, FDR = 0.043). Other decreases included *Junb* (−1.29), *Egr1* (−1.31) and *Nr4a1* (−1.21). The two doses gave similar changes (*Arc*: −1.46 at 1%, −1.62 at 10%).

In the olfactory bulb, 22 of 23 IEGs were lower (median −1.34; competitive $P = 3.8 \times 10^{-13}$), with the largest decreases in *Nr4a3* (−3.26), *Nr4a1* (−3.10), *Egr1* (−2.69) and *Fos* (−2.67). No single gene reached FDR < 0.05, as expected with three animals per group.

The self-contained label-permutation test confirmed the decrease. The observed hippocampal score was the most extreme of all 84 labellings (P = 0.012, the minimum attainable). The olfactory bulb score ranked second (P = 0.024; Fig. 2).

Genome-wide, the LA effects in the two tissues were negatively correlated (Spearman ρ = −0.32 across 11,583 genes). This indicates a strong technical component that runs in opposite directions in the two tissues. The IEG decrease occurred in both tissues and is therefore not explained by that component. Hippocampal *Bdnf* mRNA was not changed (log2FC +0.20 at 1% and +0.31 at 10%; P = 0.20).

The IEG decrease matches diazepam, not fluoxetine

Chronic diazepam decreased IEGs in the hippocampus (20/23; $P = 1.8 \times 10^{-4}$) and amygdala (19/23; $P = 6.8 \times 10^{-6}$), but not in the cerebral cortex (13/23; P = 0.38). Chronic fluoxetine did not decrease IEGs in the dentate gyrus. GSE118669 showed no bias (14/24; P = 0.17). In GSE43261, fluoxetine responders had increased IEGs (0/24 decreased; median log2FC +0.98; $P = 7.0 \times 10^{-8}$).

Acute stress and arousal move the same genes in the opposite direction, but chronic stress does not

Handling/novelty, restraint and cold swim each increased all 24 IEGs in the hippocampus at 45 min ($P < 10^{-16}$ for each; Table 2). Sleep deprivation for 5 h also increased them (4/23 decreased; $P = 9.0 \times 10^{-8}$). The genes that LA lowered are therefore the same genes that acute stressors and extended wakefulness raise, and the directions are opposite. In the rat hypothalamus, 2 h restraint likewise increased IEGs (12/13; $P = 1.2 \times 10^{-5}$) and all 10 glucocorticoid-responsive genes ($P = 5.0 \times 10^{-8}$).

Chronic stress gave the opposite picture to acute stress. After 4 weeks of chronic mild stress, IEGs were lower in all four regions examined: hippocampus and cerebral cortex 24 of 24 genes decreased, prefrontal cortex 21 of 24, amygdala 17 of 24 (competitive $P = 2.2 \times 10^{-12}$, 4.9×10^{-13}, 1.1×10^{-9} and 7.5×10^{-4}; label-permutation P = 0.050 in each region, the minimum attainable with three animals per group). The direction of the IEG programme therefore depends on the duration of the stressor: acute stressors raise it, weeks of stress lower it. The decrease after LA matches the chronic pattern as well as it opposes the acute one, and the two cannot be told apart by direction alone.

Similarity to diazepam does not extend beyond IEGs

We tested 78 diazepam-regulated hippocampal genes outside the IEG set (8 increased, 70 decreased) that were also measured in the LA data. Their direction was not reproduced in the LA hippocampus (P = 0.083) or olfactory bulb (P = 0.82).

Exploratory: GABA_A receptor subunit transcripts

This analysis was not pre-specified. We examined 11 GABA_A receptor subunit genes and five GABA-related genes (*Gad1, Gad2, Slc32a1, Tspo, Dbi*). In the LA hippocampus, *Gabra2* mRNA was higher after LA at both doses (log2FC +1.04 at 1% and +1.17 at 10%; moderated t = 3.97; label-permutation one-sided P = 0.024; genome-wide FDR = 0.18). *Gabrb3* and *Gabrb1* changed in the same direction (Table 3).

Table 3. GABA_A subunit genes with the largest changes in the LA hippocampus (exploratory)

Gene	LA hippocampus log2FC, 1% / 10%	Label-permutation P	FDR	LA olfactory bulb log2FC	Diazepam hippocampus log2FC
Gabra2 (α2)	+1.04 / +1.17	0.024	0.18	−0.21	−0.12
Gabrb3 (β3)	+0.72 / +0.81	0.036	0.19	−0.24	+0.04
Gabrb1 (β1)	+0.33 / +0.49	0.036	0.27	−0.18	+0.09
Gabra5 (α5)	+0.46 / +0.67	0.13	0.42	−0.13	−0.05

Label-permutation P is one-sided for an increase (83 relabellings; minimum attainable P = 0.012).

This observation has five weaknesses:

1. It was found after inspecting the data and does not survive genome-wide correction (FDR = 0.18).
2. It depends on two animals. One vehicle mouse (log2 signal 13.45) lay within the LA range (13.44–14.59); the other two were lower (12.63 and 12.51).
3. Several subunits rose together (*Gabra2*, *Gabrb3*, *Gabra5*). This pattern is also expected from differences in dissection or neuronal content between samples.
4. It was not seen in the olfactory bulb (−0.21) or after diazepam in the hippocampus (−0.12; P = 0.34).
5. A change in subunit mRNA does not show a change in receptor number, composition or function.

Exploratory: stress-axis and oxytocin-related transcripts

We also examined 20 genes of the hypothalamic–pituitary–adrenal axis and of neuropeptide systems implicated in stress and sleep–wake control. This analysis was not pre-specified. Four genes reached a label-permutation P of 0.024 or below (Table 4). Three move in the direction an attenuated stress response predicts; the fourth, *Fkbp5*, does not.

Table 4. Stress-axis and oxytocin-related genes after LA inhalation (exploratory)

Gene	Tissue	log2FC, 1% / 10%	Label-permutation P	FDR
Crh	Hippocampus	−0.40 / −0.40	0.012	0.26
Crh	Olfactory bulb	−0.27 / −0.46	0.024	0.96

Gene	Tissue	log2FC, 1% / 10%	Label-permutation P	FDR
Crhr1	Hippocampus	−0.73 / −0.57	0.071	0.27
Oxtr	Olfactory bulb	+0.69 / +0.35	0.024	0.96
Nr3c1	Olfactory bulb	+0.46 / +0.53	0.024	0.96
Fkbp5				

Label-permutation P is one-sided in the observed direction (83 relabellings; minimum attainable P = 0.012).

Crh encodes corticotropin-releasing hormone, the apex signal of the stress axis, and was lower in both tissues at both doses. *Oxtr*, the oxytocin receptor, was higher in the olfactory bulb. Oxytocin signalling has been linked to stress buffering, and hypothalamic *Oxtr* induction has been reported after chronic stress, so the direction here is of interest but was measured in a different region. Two transcripts of the glucocorticoid receptor system were also higher in the olfactory bulb: *Nr3c1*, the receptor itself, and *Fkbp5*, which reached the smallest attainable label-permutation P. *Fkbp5* is induced by glucocorticoids, so its increase is not what an attenuated stress response predicts; it is reported here because the two genes move together and because *Fkbp5* induction lowers receptor sensitivity, which is a different account of the same measurement. None of these genes passed genome-wide correction, the analysis followed inspection of the data, and each group contained three animals. They are reported as hypotheses for replication, not as findings.

Linalool in rat hypothalamus gives inconsistent IEG effects

Restraint for 2 h increased hypothalamic IEGs (GSE19589: 12/13 increased; $P = 1.2 \times 10^{-5}$) and all 10 glucocorticoid-responsive genes ($P = 5.0 \times 10^{-8}$). Relative to restraint alone, linalool plus restraint tended to raise IEGs in GSE19589 (P = 0.086) but lowered them in GSE90923 (P = 0.005). Fisher's combined one-sided P for a decrease was 0.032, but the two datasets disagree in direction. Linalool did not reduce glucocorticoid-responsive genes in either dataset (P = 0.20 and 0.16; combined P = 0.14). Linalool alone, without stress, did not change IEGs (P = 0.80 for a decrease).

Table 2. IEG set across all contrasts

Contrast	GEO	IEGs detected	Decreased	Median log2FC	Median statistic	Competitive P
LA vs vehicle, mouse hippocampus	GSE316912	22	21	−0.63	−3.37	9.8×10^{-12}

Contrast	GEO	IEGs detected	Decreased	Median log2FC	Median statistic	Competitive P
LA vs vehicle, mouse olfactory bulb	GSE316919	23	22	−1.34	−3.20	3.8×10^{-13}
Diazepam, mouse hippocampus	GSE76700	23	20	−0.16	−1.27	1.8×10^{-4}
Diazepam, mouse amygdala	GSE76700	23	19	−0.26	−1.66	6.8×10^{-6}
Diazepam, mouse cerebral cortex	GSE76700	23	13	−0.01	−0.07	0.38
Fluoxetine, mouse dentate gyrus	GSE118669	24	14	−0.10	−0.56	0.17
Fluoxetine responders, mouse dorsal DG	GSE43261	24	0	+0.98	+1.86	7.0×10^{-8}
Sleep deprivation 5 h, mouse hippocampus	GSE33302	23	4	+0.12	+1.89	9.0×10^{-8}
Chronic mild stress 4 wk, mouse hippocampus	GSE151807	24	24	−0.58	−8.64	2.2×10^{-12}
Chronic mild stress 4 wk, mouse cerebral cortex	GSE151807	24	24	−0.65	−7.57	4.9×10^{-13}
Chronic mild stress 4 wk, mouse prefrontal cortex	GSE151807	24	21	−0.81	−12.49	1.1×10^{-9}
Chronic mild stress 4 wk, mouse amygdala	GSE151807	24	17	−0.26	−3.10	7.5×10^{-4}
Cold swim, mouse hippocampus	GSE100236	24	0	+0.86	+5.79	7.2×10^{-17}
Restraint 30 min, mouse hippocampus	GSE100236	24	0	+0.84	+7.38	8.3×10^{-17}
Handling/novelty, mouse hippocampus	GSE100236	24	0	+0.60	+4.65	8.3×10^{-17}
Restraint 2 h, rat hypothalamus	GSE19589	13	1	+0.39	+1.44	1.2×10^{-5}
Linalool + restraint vs restraint, rat hypothalamus	GSE19589	13	2	+0.46	+2.03	1.3×10^{-3}
Linalool alone, rat hypothalamus	GSE19589	13	11	−0.15	−0.56	0.67

Statistic = moderated t (microarrays) or signed z (GSE100236). Competitive P = two-sided Mann–Whitney test of set genes against all other genes; it does not account for inter-gene correlation. The label-permutation P values are given in the text.

Discussion

Repeated LA inhalation was followed by a coordinated decrease of the IEG programme in both the hippocampus and olfactory bulb. Because every animal met a tail suspension test immediately before sampling, the comparison is between two groups that both encountered an acute stressor, and the decrease is best read as a blunted transcriptional response to that stressor. The same genes rose after every acute stressor we examined and after sleep deprivation. LA moved them the other way.

This places the effect upstream of sleep rather than inside the sleep-generating circuitry. An odorant that lowers stress-evoked activation would be expected to shorten sleep latency in an animal kept awake by stress, while doing little in an unstressed animal. That prediction is consistent with the rat hypothalamic data, in which linalool alone, without a stressor, changed neither IEGs (P = 0.80) nor the sleep–wake neuropeptides *Hcrt*, *Pmch* and *Gal*. It also fits reports that bergamot oil attenuates the corticosterone response to a stressor [3] and the lower serum corticosterone reported in the LA study itself [6].

That reading has a clear limit. Four weeks of chronic mild stress lowered the same genes in all four regions examined, as LA did. Direction alone therefore cannot separate "the stress response was blunted" from "the animal is in a chronically stressed state", because both end in less IEG transcription. Two features of the LA experiment argue against the second reading but do not exclude it: vehicle and LA animals received the identical 7-day tail suspension schedule, so any stress of the procedure was shared, and the LA groups did not differ from vehicle in body weight [6]. A study that applies the odour on a background of chronic stress, as models of stress-induced sleep disruption do, cannot rely on IEG direction alone and will need behavioural and sleep measurements alongside it.

The original study grouped *Fos*, *Junb*, *Egr1–3* and *Arc* into a module labelled kinase and transcription-factor activation and presented it as support for BDNF–TrkB signalling [6]. The deposited data show that these genes are decreased. Hippocampal *Bdnf* mRNA was unchanged, although the authors reported increased BDNF protein. The module is therefore better read as reduced stress-evoked transcription than as neurotrophin-driven induction.

Which mechanism produces that reduction is not settled by these data. Chronic diazepam lowered the same genes in the hippocampus and amygdala, and fluoxetine did not, which is compatible with a GABAergic action. But the resemblance to diazepam stopped at the IEG set: diazepam-regulated genes outside it were not reproduced (P = 0.083 in the hippocampus). IEG suppression is a common endpoint of reduced neuronal activity from many causes. The specific link to benzodiazepine-sensitive GABA_A receptors rests on behavioural pharmacology, where flumazenil abolished the anxiolytic effect of linalool odour and anosmia prevented it [5]. Flumazenil blocks ligands at the

benzodiazepine site rather than GABA release itself, so its efficacy implies that some ligand engages that site downstream of olfactory input. Endogenous site ligands, low brain levels of the odorant [8] or its metabolites, and residual activity of flumazenil remain possible explanations.

The exploratory increase in hippocampal *Gabra2* mRNA is of interest because α2-containing GABA_A receptors mediate the anxiolytic action of benzodiazepines. We do not interpret it further. It was not pre-specified, does not pass genome-wide correction, rests largely on two vehicle animals and co-occurs with other subunits in a pattern compatible with dissection differences. It should be tested in an independent cohort, preferably with region-matched qPCR and receptor-binding measurements.

The exploratory decrease in *Crh* and increase in *Oxtr* point in the same direction as the main result, and the increases in *Nr3c1* and *Fkbp5* point to the glucocorticoid receptor system as a third place to look. None survives genome-wide correction and all followed inspection of the data. We note them because they are directly testable: if LA acts by lowering stress-evoked drive, CRH signalling, oxytocin sensitivity and glucocorticoid receptor signalling are the three nodes where that should be visible, and all three can be measured in an independent cohort. The glucocorticoid node is the most informative of the three, because the direction observed here is the opposite of what reduced corticosterone exposure would produce, so a replication that measured corticosterone alongside *Fkbp5* would distinguish an attenuated stress response from an altered receptor set-point.

The rat hypothalamic datasets, in which a stressor was also applied, did not reproduce a consistent effect. Linalool plus restraint raised IEGs in one dataset and lowered them in the other. Neither dataset showed reduced glucocorticoid-responsive genes. Compound (linalool rather than LA), species, region, stressor (2 h restraint rather than a 6-min test) and normalisation all differed, and we could not renormalise the raw data. These results argue against generalising the LA finding to linalool or to the hypothalamus without new data.

These findings give testable predictions for aroma studies of stress-related sleep disturbance, in which a stressor is applied and sleep is then measured.

1. Bergamot or LA exposure should reduce c-Fos-positive cells in the olfactory bulb, hippocampus and amygdala of acutely stressed animals. This is distinct from direct activation of sleep-promoting neurons in the ventrolateral and median preoptic nuclei, which would increase c-Fos there. Staining both sets of regions in the same sections separates the two accounts.
2. The effect should be larger in stressed than in unstressed animals. An intervention that works on stress-evoked arousal should have little to act on in a calm animal.

3. If the benzodiazepine site is required, flumazenil pretreatment should abolish both the c-Fos reduction and any shortening of sleep latency.
4. The exploratory *Crh*, *Oxtr* and *Fkbp5* changes can be tested directly by region-matched qPCR with circulating corticosterone measured in the same animals, and by asking whether an oxytocin receptor antagonist blocks the effect of the odour on sleep latency in stressed animals.

Limitations

- Each LA group had three animals, and only *Arc* passed FDR < 0.05; conclusions rest on gene-set tests. The exploratory single-gene observations were not corrected for the number of genes inspected.
- The LA vehicle group differed from LA groups in odour exposure only, but sampling time of day was not reported. Clock-output genes differed (hippocampal *Dbp* +1.03 log2, $P = 2 \times 10^{-5}$), so a time-of-sampling difference cannot be excluded.
- The diazepam data come from chronic treatment of unknown timing relative to the last dose. No acute benzodiazepine or Z-drug brain transcriptome was found for comparison.
- The GEO overall-design field of GSE316912 describes a different experiment (SH-SY5Y cells treated with oleacein); we relied on the published methods [6].
- Bergamot oil itself has no public brain transcriptome. Inferences about bergamot depend on its LA content.
- No public dataset combines odorant exposure with recorded sleep. The link from attenuated stress-evoked transcription to sleep onset is an inference and was not measured here.
- IEG suppression is not specific to relief from stress. Chronic mild stress produced the same direction, so the measure cannot by itself separate an attenuated stress response from a chronically stressed state.
- The chronic stress comparison rests on three animals per group per region, and the deposited matrix is median-centred, so absolute expression levels were not available within that dataset.

Declarations

Data availability: All data are public in GEO under the accessions in Table 1. Analysis scripts are archived at Zenodo, doi:10.5281/zenodo.24119697.

Author contributions: K.O. is the sole author. K.O. designed and performed the reanalysis and wrote the manuscript.

Use of AI tools: Data retrieval, statistical scripts and a first draft of the text were prepared with assistance from an AI system (Claude, Anthropic). The author checked all analyses and takes full responsibility for the content.

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Competing interests: The author declares no competing interests.

Figure legends

Figure 1. The IEG programme across public datasets, grouped by intervention. Log2 fold change versus control for 16 representative IEGs, grouped by intervention. Acute stress and arousal: cold swim, restraint and handling/novelty at 45 min (GSE100236, n = 5 vs 5 each) and 5 h sleep deprivation (GSE33302, n = 8 vs 9). Chronic stress: 4 weeks of chronic mild stress in four regions (GSE151807, n = 3 vs 3 per region). Linalyl acetate: pooled 1% and 10% inhalation, sampled immediately after a tail suspension test (n = 6 vs 3). Diazepam: 10 days of treatment in hippocampus, amygdala and cortex (n = 3 vs 3). Fluoxetine: dentate gyrus (GSE118669, n = 8 vs 8; GSE43261 responders, n = 7 vs 8). Blank cells: gene not detected after filtering.

Figure 2. Sample-label permutation test of the IEG decrease after LA. Grey histograms show the IEG score for all 83 alternative labellings of the nine samples (3 vs 6). The red line shows the observed score. One-sided P = (k + 1)/84.

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Figures

Figure 1

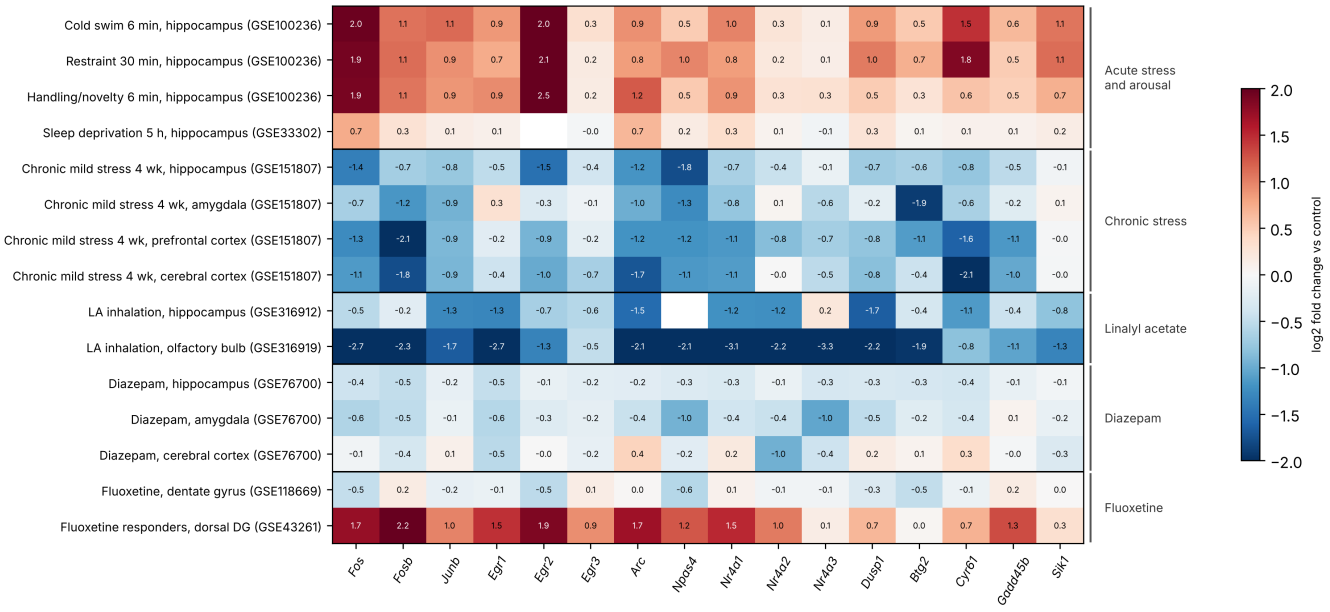
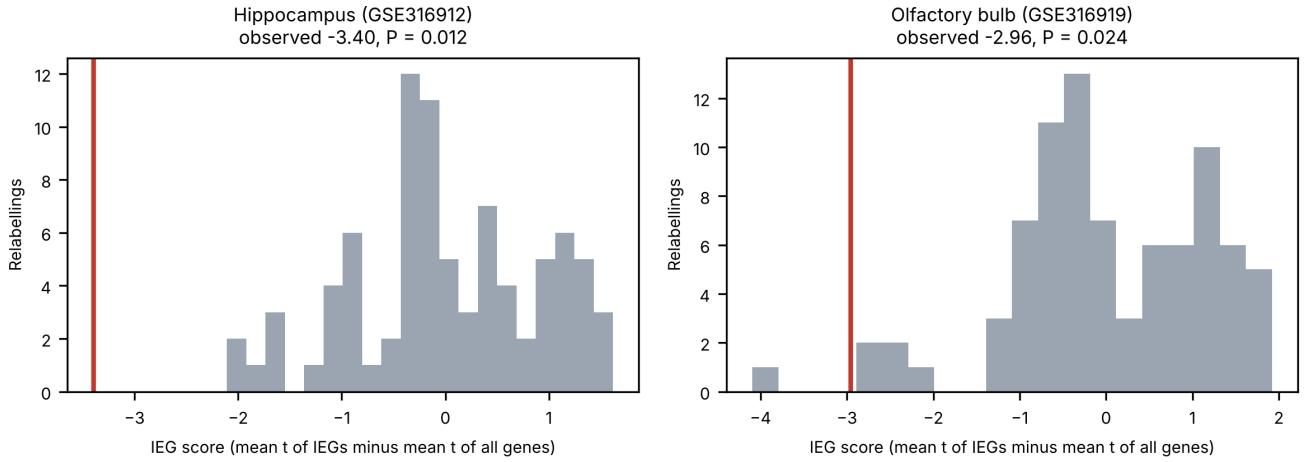

Cold swim 6 min, hippocampus (GSE100236)
Restraint 30 min, hippocampus (GSE100236)
Handling/novelty 6 min, hippocampus (GSE100236)
Sleep deprivation 5 h, hippocampus (GSE33302)
Chronic mild stress 4 wk, hippocampus (GSE151807)
Chronic mild stress 4 wk, amygdala (GSE151807)
Chronic mild stress 4 wk, prefrontal cortex (GSE151807)
Chronic mild stress 4 wk, cerebral cortex (GSE151807)
LA inhalation, hippocampus (GSE316912)
LA inhalation, olfactory bulb (GSE316919)
Diazepam, hippocampus (GSE76700)
Diazepam, amygdala (GSE76700)
Diazepam, cerebral cortex (GSE76700)
Fluoxetine, dentate gyrus (GSE118669)
Fluoxetine responders, dorsal DG (GSE43261)
Acute stress and arousal
Chronic stress
Linalyl acetate
Diazepam
Fluoxetine
Fos
FosB
Junb
Egr1
Egr2
Egr3
Arc
Npas4
Nr4a1
Nr4a2
Nr4a3
Dusp1
Btg2
Cyr61
Gadd45b
Slk1
log2 fold change vs control
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0

Figure 2


Hippocampus (GSE316912)
observed -3.40, P = 0.012
Olfactory bulb (GSE316919)
observed -2.96, P = 0.024
Relabellings
IEG score (mean t of IEGs minus mean t of all genes)