Loss of the metabolism and sleep regulating neuronal populations expressing orexin and oxytocin in the hypothalamus in amyotrophic lateral sclerosis

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Abstract

Aims: To determine the underlying cellular changes and clinical correlates associated with pathology of the hypothalamus in amyotrophic lateral sclerosis (ALS), as hypothalamic atrophy occurs in the preclinical phase of the disease.

Methods: The hypothalamus was pathologically examined in nine patients with amyotrophic lateral sclerosis in comparison to eight healthy control subjects. The severity of regional atrophy (paraventricular nucleus: PVN, fornix and total hypothalamus) and peptideergic neuronal loss (oxytocin, vasopressin, cocaine- and amphetamine-regulating transcript: CART, and orexin) was correlated with changes in eating behaviour, sleep function, cognition, behaviour and disease progression.

Results: TAR DNA-binding protein 43 (TDP-43) inclusions were present in the hypothalamus of all patients with amyotrophic lateral sclerosis. When compared to controls, there was atrophy of the hypothalamus (average 21% atrophy, \( p = 0.004 \)), PVN (average 30% atrophy \( p = 0.014 \)) and a loss of paraventricular oxytocin-producing neurons (average 49% loss \( p = 0.02 \)) and lateral hypothalamic orexin-producing neurons (average 37% loss, significance \( p = 0.02 \)). Factor analysis identified strong relationships between abnormal eating behaviour, hypothalamic atrophy and loss of orexin-producing neurons. With increasing disease progression, abnormal sleep behaviour and cognition associated with atrophy of the fornix.

Conclusions: Substantial loss of hypothalamic oxytocin-producing neurons occurs in ALS, with regional atrophy and the loss of orexin neurons relating to abnormal eating behaviour in ALS. Oxytocin- and orexin neurons display TDP43 inclusions. Our study points to significant pathology in the hypothalamus that may play a key role in metabolic and pathogenic changes in ALS.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease associated with both upper and lower motor neuron dysfunction leading to progressive muscle weakness and bulbar dysfunction. It is increasingly recognised that ALS also affects energy metabolism, eating behaviour, sleep and autonomic function. Patients with ALS have marked weight loss and low body mass index (BMI) that correlate with poor survival. Traditionally ALS has been regarded as a disease associated with malnutrition, with suggestions that nutritional intake decreases as the disease progresses. However, eating behaviour in ALS is more complex with pre-symptomatic patients and patients developing cognitive and behavioural changes that increase their caloric intake.

ALS shares strong genetic and pathological overlap with frontotemporal dementia, a syndrome in which the hypothalamus has been identified to be affected very early in the disease in association with abnormal eating behaviours. Hypothalamic atrophy is observed in ALS using volumetric MRI and is associated with lower BMI. The hypothalamus is central to the control of eating behaviour, with an appetite stimulating pathway targeting neurons of the arcuate nucleus, and an appetite suppressing pathway acting on neurons producing both pro-opiomelanocortin and cocaine- and amphetamine-related transcript (CART). Oxytocin, vasopressin and orexin neuropeptides produced in the hypothalamus also play an extensive role in the regulation of eating and other behaviours.

As TAR DNA-binding protein 43 (TDP-43) pathology is found in most ALS cases (and in the hypothalamus in one third of patients), and in patients with frontotemporal dementia those with TDP-43 pathology have the greatest hypothalamic pathology, degeneration of hypothalamic neurons may also occur early in ALS and impact abnormal eating behaviours and metabolic function, that can then potentially affect disease progression. Using serial section microscopy, the current study aimed to assess the degree of hypothalamic pathology (presence of TDP-43 inclusions, degree of atrophy and peptidergic neuronal loss) in ALS compared to healthy controls, and correlate these pathologies to changes in eating behaviour, sleep, cognition, behaviour and disease progression.

MATERIALS AND METHODS

Participants

Nine patients with ALS were recruited for longitudinal assessments and brain donation from the Forefront ALS clinic based at the Brain and Mind Centre, University of Sydney, Australia. All patients underwent neurological review, cognitive assessment and met current clinical diagnostic criteria for ALS. Patients were classified as having either limb or bulbar weakness as their first symptom. All patients were negative for the C9orf72 mutation. Cognitive and behavioural testing was conducted by trained neuropsychologists, and physical limitations were taken into account. Data from the initial assessment were used for correlations (see below). Disease duration was calculated from the date of onset until the date of death. The brain donor study protocols were approved by the South Eastern Sydney Local Health District and the University of Sydney human ethics committees.

Brain retrieval at death and pathological characterisation was performed by the Sydney Brain Bank at Neuroscience Research Australia with approval by the South Eastern Sydney Local Health District and the University of New South Wales human ethics committees. The post-mortem brain tissue was released for this study following approval of the project by their Scientific Review Committee (PID500). Tissue from control cases (N = 8) from our previously published study using the same techniques was included for comparison. These control cases had no clinical evidence of neurologic disease and were obtained previously from the Sydney Brain Bank at Neuroscience Research Australia following approval by their Scientific Committee (PID073).

Clinical assessment at initial presentation

All clinical assessments were conducted at initial presentation.

General screening

Global cognitive functioning was determined using the revised Addenbrooke’s Cognitive Examination (ACE-R), a validated screening measure of cognitive function covering attention, memory, verbal fluency, language and visuospatial abilities.

Functional assessment

Functional assessment at initial presentation was measured using the ALS functional rating scale.

Behavioural assessment

Carers rated the extent of behavioural changes in the patients via the Cambridge Behavioural Inventory (CBI), a validated measure of behavioural changes in ALS.
Body mass index (BMI)

Height and weight were measured barefoot and BMI calculated (weight in kilograms/height in metres squared).

Post-mortem tissue processing

The entire unilateral hypothalamus from the ALS cases (N = 9) were excised from formalin-fixed coronal slices and processed for immunohistochemistry. The ALS cases were compared to control cases (N = 8) prepared in the same way, as published previously. Briefly, hypothalamic tissue blocks spanning the entire hypothalamic region were first cryoprotected in 30% sucrose in 0.1 M tris buffer (pH 7.4). The blocks were then cut serially in the coronal plane using a semi-freezing microtome at a thickness of 50 μm and collected in 15 equally spaced series throughout the entire anteroposterior extent of the hypothalamus. One series was mounted on glass slides and processed for Nissl (0.5% cresyl violet or CV, ICN Biomedicals Inc, stabilised with 10% acetic acid) and myelin (0.1% luxol fast blue or LFB, Solvent Blue 38, Sigma, stabilised with lithium carbonate) staining. Free-floating sections were processed immunohistochemically using a primary antibody against oxytocin (1:10,000, made in rabbit, Phoenix Pharmaceuticals), vasopressin (1:30,000, made in rabbit, Millipore), orexin (1:30,000, made in rabbit, Phoenix Pharmaceuticals), CART (1:10,000, made in rabbit, Phoenix Pharmaceuticals), and for Tar DNA Binding Protein 43 phosho Ser409/410 (TDP-43; antibody made in rabbit, 1:2000, Phoenix Pharmaceuticals) and for Tar DNA Binding Protein 43 phosho Ser409/410 (TDP-43; antibody made in rabbit, 1:2000, Phoenix Pharmaceuticals) and for Tar DNA Binding Protein 43 phosho Ser409/410 (TDP-43; antibody made in rabbit, 1:2000, Phoenix Pharmaceuticals). The entire unilateral hypothalamus from the ALS cases (N = 9) were excised from formalin-fixed coronal slices and processed for TDP-43 and counterstained with CV. Two investigators (ÅP, SG) assessed TDP-43 immunopositive staining in the lateral hypothalamic area, paraventricular nucleus (PVN) and the fornix. For confocal analyses of co-localisation of TDP-43 inclusions and orexin or oxytocin immunofluorescence, confocal images were acquired using a Nikon Eclipse 90i microscope (Nikon, Instruments Inc., Europe B.V.) coupled to a Nikon D-Eclipse C1 laser scanning confocal unit equipped with a 488-nm sapphire diode laser, a 543-nm argon-ion laser (CVI Melles Griot, Albuquerque, NM, USA) and 639-nm cube diode laser (Coherent Inc.). Confocal Z-stacks were obtained with a 60×Apochromat oil-immersion objective with laser excitation in sequential mode using nis-element ar software version 4.0. Confocal images with orthogonal projections were then generated using the nis-element analysis software version 4.5. Estimation of the number of cells with TDP-43 inclusions and oxytocin or orexin immunofluorescence was made on 20 randomly selected confocal Z-stack images using a 60× Plan-Apo oil objective (numerical aperture = 1.4) in each of the PVN and LHA in 4 ALS cases. For each image, first the number of oxytocin or orexin immunopositive cells was determined and then the number of cells which showed overlap with TDP-43 was determined. Between 97–135 cells were evaluated for each case.

Due to limitations of the tissue, detailed quantification using stereological analyses of the number of neuropeptide-expressing cells was performed on seven to eight cases with ALS and compared with four control cases. In these cases, the volumes of the entire hypothalamus, the fornix within the hypothalamic region, and the PVN were determined on sections stained for CV/LFB using the CAST module in the VIS software (Visiopharm) and applying the optical fractionator using the Cavalieri method. As previously described,25 the border of the hypothalamus was outlined in each section between bregma -1.3 to 12 mm and the PVN between bregma 00 and 6.7 mm (based on the Atlas of the Human Brain).25 The cross-sectional areas of the hypothalamus, the fornix and the PVN were then computed, and the volumes were determined by multiplying the cross-sectional areas by the distance between sections (750 μm). The average number of slides assessed per ALS case was 12 ± 2 (mean ± SD), and 12 ± 2 per control case for hypothalamic volume estimations. The average number of slides assessed for the PVN volume estimations per ALS case was 7 ± 1 and 6 ± 1 per control case, and for the fornix 9 ± 1 per ALS case and 8 ± 2 per control case.

Estimates of the total number of immunopositive cells were obtained with an unbiased stereological quantification method by employing the optical fractionator principle on blind-coded slides. Neuropeptide-containing neurons were quantified with the Computer Assisted Toolbox Software (CAST) module in VIS software (Visiopharm,
Horsholm, Denmark), or post-processed in Metafer software package (Althaussen, Germany) and transferred to the CAST module in the VIS software (Visiopharm, Horsholm, Denmark), using a 60x Plan-Apo oil objective (numerical aperture=1.4) on a Nikon 80i or Zeiss microscope equipped with an x-y axis motorised stage and a high precision linear encoder on the z-axis motor (Heindenhain, Traunreut, Germany). Sampling for the quantitation was initiated with a random position placement of the counting frame, which then moved systematically throughout all the sampling positions with a specifically chosen x-y step length until the entire region of interest was sampled. The x-y step length was adjusted accordingly to achieve maximal sampling and to minimise the coefficient of error, which would usually yield roughly between 150 and 400 neurons per brain for the different neuropeptide staining. The total cell numbers in each case were estimated using the following formula based on the optical fractionator principle: \( N = \Sigma Q \times \left( \frac{x \times y \text{ step length}}{\Sigma Q} \right) \times \text{number of series} \). Where \( N \) is equal to the total number of neurons, \( \Sigma Q \) is the number of neurons counted per brain. For any of the stains assessed, there was no significant difference in the average number of slides per case.

### Statistical analyses

Analyses were conducted using IBM SPSS statistics (version 24.0) and \( p < 0.05 \) regarded as significant. Kolmogorov-Smirnov tests were run to determine suitability of variables for parametric analyses. Mann-Whitney U tests and Fisher exact tests were used to evaluate group differences in demographic and clinical variables, and hypothalamic volumes and pathologies. A principal component factor analysis was performed to determine relationships between continuous clinical and pathological variables. A principal component factor analysis was performed to determine relationships between continuous clinical and pathological variables. The number of factors in the covariance/correlation matrix was selected by the criterion of an eigenvalue >1, and variables were considered relevant if they had factor loadings stronger than ±0.65.27

### Data sharing statement

The authors are happy to make all data and statistical plans available upon reasonable request until 2025.

### RESULTS

#### Demographic data

Samples from nine ALS patients were examined and compared to eight control subjects (demographics in Table 1). There was no significant difference in gender (\( p = 0.201 \)) or age (\( p = 0.089 \)) between these groups. Cognitive and behavioural results for the ALS group are presented in Table 1 and are representative of changes noted in ALS.2 The average post-mortem time was 23 ± 18 h (mean ± SD) for the ALS cases compared to 16 ± 11 h for control cases (Table S1).

#### Confirming hypothalamic atrophy in ALS

Hypothalamic atrophy (at onset and even pre-symptomatically) has been reported in ALS using volumetric analyses of MR images and correlates with BMI.22 We confirmed a reduction in hypothalamic volume in ALS (average 21% atrophy compared to controls) using stereological analyses of post-mortem brain tissue (Figure 1A-C, Table 2). The PVN is one area within the hypothalamus that regulates metabolism, and our data showed atrophy of the PVN in ALS (average 30% atrophy compared to controls) (Figure 1D-F, Table 2). Multiple regression analyses with hypothalamic volume or PVN volume as dependent variables, age and sex as independent variables showed significant differences between ALS and control cases, but no significant effects of age or sex (Supplementary statistical analyses 1). We found no significant correlation between the volumes of the hypothalamus and the PVN with post-mortem delay time (\( r = 0.086, p = 0.761 \) and \( r = 0.088, p = 0.754 \) respectively. There was variability but no consistent reduction in the volume of the fornix (Table 2).

#### Confirming TDP-43 inclusions in the hypothalamus in ALS

The formation of intracellular inclusions of TDP-43 is a hallmark of pathology in ALS and TDP-43 inclusions have been found in the

| Table 1 | Clinical and demographic variables (average±standard deviation) |
|---------|---------------------------------------------------------------|
| Variable | ALS | Control | Group comparison |
| Male: Female | 4:5 | 6:2 | Fisher exact test \( U = 18, p = 0.108 \) |
| Age at death (y) | 71 ± 9 | 61 ± 14 | Mann-Whitney U test \( U = 18, p = 0.861 \) |
| Disease duration at death (y) | 3.0 ± 2.1 | NA | NA |
| Time from testing to death (y) | 1.8 ± 1.1 | NA | NA |
| Limb:Bulbar | 7.3 | NA | NA |
| BMI (m²) | 25 ± 2.5 | NA | NA |
| ALSFRS (/40) | 31 ± 12 | NA | NA |
| ACE-R (/100) | 92 ± 10 | NA | NA |
| CBI total | 32 ± 23 | NA | NA |
| CBI eating | 2.0 ± 1.4 | NA | NA |
| CBI stereotypical behaviour | 2.2 ± 2.3 | NA | NA |
| CBI sleep | 3.3 ± 2.0 | NA | NA |
| CBI motivation | 3.7 ± 2.0 | NA | NA |
| CBI abnormal behaviour | 1.3 ± 1.9 | NA | NA |

Abbreviations: ACE-R, revised Addenbrooke’s cognitive examination; ALS, amyotrophic lateral sclerosis; ALSFRS, ALS functional rating scale; BMI, body mass index; CBI, Cambridge behavioural inventory.

\( a \)Variables used in the factor analysis; \( b \)Item subscores also included in the factor analysis.
We assessed the presence of TDP-43 inclusions (Figure 2) in the PVN, lateral hypothalamus and fornix in ALS. We found that TDP-43 immunopositive intracellular inclusions were present in all these regions in all ALS cases. The frequency of the inclusions was <1% of cells.

Selective loss of oxytocin- and orexin-containing neurons in ALS

We then investigated if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism. We assessed if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism. We assessed if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism. We assessed if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism. We assessed if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism.

Table 2: Hypothalamic volumes and neuronal numbers (average ± standard deviation)

| Measures | ALS       | Control   | U test | p value |
|----------|-----------|-----------|--------|---------|
| Volumes  |           |           |        |         |
| Brain (ml) | 1329 ± 194 | 1429 ± 133 | 19     | 0.114   |
| Hypothalamus (mm$^3$) | 321 ± 59     | 406 ± 46   | 4.5    | 0.004   |
| PVN (mm$^3$) | 11 ± 2       | 15 ± 5    | 9.0    | 0.015   |
| Fornix (mm$^3$) | 47 ± 6       | 54 ± 11   | 18.5   | 0.167   |
| Neuronal number |          |           |        |         |
| Oxytocin*  | 13,526 ± 6304 | 26,753 ± 6194 | 2.0   | 0.024   |
| Vasopressin* | 31,809 ± 10,928 | 34,813 ± 9420 | 13.5  | 0.711   |
| CART*      | 27,675 ± 11,470 | 29,550 ± 16,286 | 15.0  | 0.933   |
| Orexin     | 22,371 ± 7887 | 35,550 ± 6491 | 2.0   | 0.024   |

Abbreviations: CART, cocaine- and amphetamine-regulating transcript; PVN, paraventricular nucleus.
*Variables used in the factor analysis.

We then investigated if there was a reduction in the number of the peptidergic containing neurons (oxytocin-, vasopressin-, CART- and orexin) in the hypothalamus as these peptides also play a role in eating behaviour and metabolism. Oxytocin and vasopressin are produced in the PVN and supraoptic nucleus, with vasopressin also produced in the suprachiasmatic nucleus. CART is produced in the PVN, arcuate nucleus, dorsomedial and lateral hypothalamus and orexin found in the lateral hypothalamus.

Quantitation of PVN neurons found no loss of vasopressin- (Figure 3D-F) or CART- immunopositive neurons (Figure 3G-H), but there was a reduction of 49% in the number of oxytocin-immunopositive neurons in ALS cases compared to controls (Figure 3A-C, Table 2). Quantitation of the lateral hypothalamus showed a reduction of 37% of orexin neurons in ALS cases compared to controls (Figure 4A-C, Table 2). As the orexin- and oxytocin-expressing neuronal populations were affected in ALS cases, we investigated whether the frequency of the TDP-43 inclusions would be higher in these neurons. In fact, 24 ± 6% of the orexin-expressing neurons and 33 ± 6% of the oxytocin-expressing neurons had TDP-43 inclusions as assessed in the confocal microscope (Figure 5).
Factor analysis

A factor analysis was performed to determine the interrelationships between continuous clinical and pathological variables in the ALS group (Factors included in analyses are marked in Tables 1 and 2, Supplementary statistical analyses 2, also contains correlation matrix). Two main factors containing significant loadings of clinical and pathological variables were identified accounting for 33% and 32% of the variance respectively. Factor 1 (Table 3) showed significant interrelationships between the different CBI items identifying abnormal eating behaviours, hypothalamic atrophy and the number of orexin neurons. Hypothalamic atrophy and reduced orexin neuronal numbers were related to increasing evidence of abnormal eating behaviours (Table 3). Factor 2 (Table 3) showed significant interrelationships between disease progression (measured by decreasing ALSFRS), cognitive dysfunction (reduced ACE-R), increased frequency of abnormal behaviours (CBI total frequency), including sleep behaviours (CBI sleep score) and eating the same foods (CBI same foods score), and a reduction in the volume of the fornix and the number of PVN CART neurons (even though these were not reduced overall). These abnormalities occur in concert with disease progression (Table 3).

DISCUSSION

In this comprehensive evaluation of pathological changes of the neuropeptidergic neurons of the hypothalamus in ALS, we have identified hypothalamic atrophy and a selective loss of oxytocin-producing neurons in the PVN and orexin-producing neurons in the lateral hypothalamus in ALS, with no overall change to vasopressin- and CART-producing neurons. Abnormal TDP-43 inclusions were
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also identified in the hypothalamus of all ALS cases, where around 23% of orexin neurons and 33% of oxytocin neurons were estimated to contain TDP-43 inclusions. The consistent and dramatic loss of oxytocin-producing neurons in ALS patients was not related to demographic or clinical indices. Loss of orexin-producing neurons was related to eating abnormalities, as was overall hypothalamic atrophy. This is a highly selective reduction in neuropeptidergic secreting hypothalamic neurons that would impact on the regulation of eating behaviour and metabolism in ALS (Figure 6). In addition, the milder but progressive atrophy of the fornix and the number of CART-producing neurons both correlated with abnormal sleep behaviours and cognition with clinical progression of ALS (as measured by ALSFRS). These data suggest that degeneration of select groups of peptidergic neurons in the hypothalamus significantly may impact the pathogenesis and clinical behavioural changes seen in ALS.

The specificity of these deficits in hypothalamic neuropeptide populations to function and not pathology is highlighted by the preservation of these neuronal groups in frontotemporal dementia with TDP-43 inclusions, while there are similar overall peptidergic cell changes in Huntington’s disease (see review). There is no loss of PVN oxytocin neurons with age or Alzheimer’s disease, suggesting only certain neurodegenerative diseases impact on these hypothalamic oxytocin neurons. Both ALS and Huntington’s disease have pre-symptomatic weight loss in association with systemic metabolic dysfunction and significant muscle wasting. Of direct interest is the observation that oxytocin is a myokine that activates a complex array of signalling pathways that regulate muscle homeostasis, with muscle regeneration significantly impaired in oxytocin knockout mice. Physical exercise increases hypothalamic (and plasma) oxytocin that impacts directly on muscle health, and is currently recommended for patients with Huntington’s disease, where upper and lower motor neurons are not impacted (in contrast to ALS). Enhancement of peripheral oxytocin may assist muscle wasting and energy metabolism in both these neurodegenerative disorders, although without motor neuron saving therapies, it is likely to be of less benefit to patients with ALS.

**FIGURE 4** Orexin-immunopositive neurons in ALS. The total number of neurons expressing the neuropeptide orexin (hypocretin, A-C) was assessed using stereology in immunohistochemically processed sections in one series of 15 cut through the whole hypothalamus. The data are expressed as mean ± SD (n = 4–7/group). * = p < 0.05, Mann-Whitney U-test. Scale bar in B: 400 µm.

**FIGURE 5** Presence of TDP-43 inclusions in the orexin and oxytocin neuronal populations in ALS. Representative high-power immunofluorescence images taken from human post-mortem hypothalamic tissue from ALS cases immunohistochemically processed for TDP-43 (red) and oxytocin (green, A-C) and orexin (green, D-F) respectively. Orthogonal images show TDP-43 co-localising with oxytocin and orexin neurons respectively. The images were taken by using a Nikon confocal microscope with 60X apochromat oil-immersion objective. Scale bar = 10 µm.
Near complete loss of hypothalamic orexin neurons occurs in narcolepsy, while only a mild loss of orexin neurons occurs in Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease. In Alzheimer’s disease, the loss of these neurons is associated with reduced BMI, while in Parkinson’s disease, the loss is associated with increasing severity of clinical disease and occurs in association with a mild loss of hypothalamic melanin concentrating hormone-producing neurons. Orexin-producing neurons in the lateral hypothalamus innervate both PVN oxytocin-producing as well as PVN parvocellular neurons (among other brain regions). The greater loss of oxytocin-compared with orexin-producing neurons in ALS and Huntington’s disease could suggest a retrograde degenerative change affecting the orexin-producing neuronal population, consistent with reducing BMI over time.

The orexin innervation of the PVN entrains neurons (a proportion of which are CART-producing) to the circadian rhythm for their secretion, a role orexin neurons also play in many sleep circuits. In particular, the lateral hypothalamic orexin-producing neurons innervate the paraventricular thalamus, a region critical for wakefulness control. This thalamic region also receives a dense projection from CART-containing hypothalamic neurons. Atrophy of the fornix and a loss of PVN CART-producing neurons was associated with abnormal sleep behaviours in ALS in the present study. These changes are consistent with a slow retrograde degeneration of these important and parallel orexin and CART pathways from the hypothalamus to the thalamus in ALS. Orexins and CART are important for maintaining long periods of wake and suppressing REM sleep with a reduction in their levels causing shorter than usual wake bouts and sudden transitions from wake to REM sleep. Sleep disorders are common in ALS due to both peripheral (such as muscle weakness) and central factors, with many patients complaining of disturbed sleep, daytime sleepiness and fatigue. Interestingly, ALS patients with genetic abnormalities have higher frequencies of sleep disturbances than non-mutation carriers. Furthermore, inclusions of dipeptide repeat proteins (DRP) resulting from the C9orf72 hexanucleotide repeat expansion have been found in sleep regulating pinealocytes and neurons in the suprachiasmatic nucleus of the hypothalamus in ALS cases. These neurons did not form TDP-43 inclusions. Another recent study also showed the presence of DRP aggregates in hypothalamic nuclei regulating the hypothalamic-pituitary axis such as the PVN, the arcuate nucleus and the periventricular nucleus. Growth factors in the hypothalamic-pituitary axis have been found to be reduced in the cerebrospinal fluid of ALS patients, and these growth factors are involved in sleep regulation partly through orexin neurons. Taken together, emerging studies point to novel findings of pathology in the hypothalamus that may have implications for sleep disturbances in ALS.

Interestingly, both oxytocin and orexin are neuropeptides involved in the regulation of emotions and social behaviour.
Huntington disease, where orexin and oxytocin loss are also prominent, psychiatric and behavioural change predate the motor disorder by up to 15 years (see review). Extensive research in frontotemporal dementia shows emotion processing deficits, and clinical trials have been conducted to examine the effect of oxytocin in mediating behavioural changes. There is emerging evidence in ALS suggesting that emotion processing deficits and behavioural changes may also be present, particularly as ALS patients develop cognitive changes and signs of frontotemporal dementia, but may also occur prior to motor neuron involvement. Although no correlations were detected between orexin and oxytocin-producing cell numbers and emotional behaviour in the present study, increasing atrophy of the fornix did correlate with increasing behavioural changes as measured on the CBI. Further studies are needed to investigate the extent of behavioural change in ALS and its relation to hypothalamic atrophy and the effects on the orexin and oxytocin system.

Major strengths of the current study include in depth stereological examination of the hypothalamus in a clinically well phenotyped cohort. One weakness is that it was conducted in a relatively small cohort. The reason for this is that fixed entire hypothalamis, that allow for stereological analyses of the whole region, as well as entire hypothalamic nuclei from ALS cases are very rare in brain banks. Future studies will require replication in larger cohorts and the development of methods to examine hypothalamic and peptidergic function in living cohorts. One of the limitations of this study is that correlations relied on carer surveys, which were conducted at first clinical presentation, a mean time of 1.8 years from patient’s death. Whilst these carer surveys have been shown to correlate to the abnormal behaviours targeted, they need to be interpreted with caution. Also, ideally clinical assessments should be conducted as close to the time of death as possible, but this is often problematic due to difficulties of patients with more burdensome symptoms participating in clinical assessments. Future studies should assess hypothalamic changes in relation to validated methods of assessing eating behaviour and sleep function. It would also be interesting to assess the underlying mechanisms of selective loss of orexin- and oxytocin-producing neurons in ALS as well as direct relationships to phenotypic consequences using (chemo)genetic manipulations in experimental models of the disease. Furthermore, it would be important to compare the identified hypothalamic changes to those identified in other conditions with weight loss as part of disease progression (e.g., in cachexia related to cancer and heart disease). Further studies should also examine hypothalamic changes in patients with SOD-1 associated ALS, where the underlying pathology is well known, as well as in other forms of motor neuron disease (including primary lateral sclerosis).

This study has assessed neuropeptidergic-producing neurons in the hypothalamus of ALS patients compared to healthy controls. The data confirm atrophy of the hypothalamus in ALS that correlates with changes in eating behaviour and using serial sections, we show consistent TDP-43 pathology in all hypothalamic areas assessed, with a relatively high frequency in remaining orexin- and oxytocin-producing neurons. We identify a considerable, selective loss of PVN oxytocin neurons likely to contribute to energy metabolism dysfunction and muscle wasting. We also found a smaller loss of orexinergic neurons that, in association with subsequent atrophy of the fornix and loss of CART-producing neurons, are likely to contribute to increasing eating, sleep and cognitive disturbances in ALS over time. Further studies are required to ascertain the effect that changes in hypothalamic peptides may have on muscle strength and disease progression in ALS. Future studies should also focus on the timing of the development of these hypothalamic changes in ALS and whether they occur pre-symptomatically in genetic at-risk cohorts. Importantly, further research is required to ascertain whether these neuropeptidergic pathways may be amenable to replacement therapies and provide novel targets for the development of therapies aiming at preventing or at least modifying disease progression in ALS.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests. Sanaz Gabery: Reports no disclosures. Rebekah Ahmed: Reports no disclosures. Jashelle Caga: Reports no disclosures. Matthew C Kiernan: Reports no disclosures. Glenda Halliday: Reports no disclosures. Asa Petersen: Reports no disclosures.
AUTHOR CONTRIBUTIONS
Sanaz Gabery performed the experiments and analysed the data, and involved in manuscript preparation. Rebekah M Ahmed contributed to study concept, clinical phenotyping, data analyses, manuscript preparation and writing. Jashelle Caga contributed to data analyses, manuscript preparation and writing. Matthew C Kiernan contributed to data analyses, manuscript preparation and writing. Glenda Halliday contributed to study concept, data analyses, manuscript preparation and writing. Asa Petersen contributed to study concept, pathological assessment, data analyses, manuscript preparation and writing.

ETHICS APPROVAL
The brain donor study protocols were approved by the South Eastern Sydney Local Health District and the University of Sydney human ethics committees. Brain retrieval at death and pathological characterisation was performed by the Sydney Brain Bank at Neuroscience Research Australia with approval by the South Eastern Sydney Local Health District and the University of Sydney human ethics committees. The post-mortem brain tissue was released for this study following approval of the project by their Scientific Review Committee (PID500). Tissue from control cases (N = 8) from our previously published study using the same techniques was included for comparison. These control cases had no clinical evidence of neurologic disease and were obtained previously from the Sydney Brain Bank at Neuroscience Research Australia following approval by their Scientific Committee (PID073).

PEER REVIEW
The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12709.

DATA AVAILABILITY STATEMENT
The authors are happy to make all data and statistical plans available upon reasonable request until 2025.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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