Neuropeptide depletion in the amygdala in SUDEP: a post mortem study

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Abstract

Objective: Sudden unexpected death in epilepsy (SUDEP) is typically unwitnessed but can be preceded by seizures in the period prior to death. Peri-ictal respiratory dysfunction is a likely mechanism for some SUDEP and central apnoea has been shown following amygdala stimulation. The amygdala is enriched in neuropeptides that modulate neuronal activity and can be transiently depleted following seizures. In a post mortem SUDEP series we sought to investigate alterations of neuropeptidergic networks in the amygdala, including cases with recent poor seizure control.

Methods: In 15 SUDEP cases, 12 epilepsy controls and 10 non-epilepsy controls we quantified the labelling index (LI) for galanin, neuropeptide Y (NPY) and somatostatin (SST) in the lateral, basal, accessory basal nuclei and peri-amygdala cortex with whole slide scanning image analysis. Within the SUDEP group, 7 had recent generalised seizures with recovery 24 hours prior to death (SUDEP-R).

Results: Galanin, NPY and SST LI were significantly lower in all amygdala regions in SUDEP cases compared to epilepsy controls ($p<0.05$ to <0.0005) and galanin LI lower in the lateral nucleus compared to non-epilepsy controls ($p<0.05$). There was no difference in the LI in SUDEP-R group compared to other SUDEP. Higher LI was noted in epilepsy controls than non-epilepsy controls, significant for NPY in lateral and basal nuclei ($p<0.005$ and $p<0.05$).

Significance: A reduction in galanin in the lateral nucleus in SUDEP could represent acute depletion, relevant to post-ictal amygdala dysfunction. In addition, increased amygdala neuropeptides in epilepsy controls supports their seizure-induced modulation that is relatively deficient in SUDEP; this could represent a vulnerability factor for amygdala dysfunction in the post-ictal period.

KEY WORDS: Amygdala, lateral nucleus, SUDEP, galanin, neuropeptide Y, somatostatin

Introduction

Post-ictal central apnoea is regarded as a clinical biomarker for SUDEP risk [1]. In the MORTEMUS study of witnessed and monitored SUDEP, post-ictal respiratory depression was recorded [2] but the physiological basis for the respiratory depression is as yet unclear. There is a growing body of evidence, however, for altered and abnormal connectivity of higher central autonomic centres, including the amygdala, regulating autonomic and voluntary respiratory dysfunction during a seizure [3]. Volumetric studies show increased right-sided amygdala grey matter volumes [4]. Ictal central apnoea can arise with seizure spread to the amygdala [5] and several studies have demonstrated apnoea following electrode stimulation of the amygdala in people with epilepsy undergoing investigations [6-8]. One interpretation of these findings is that amygdala networks influence breathing through downstream activation of cortical or extended amygdala regions connected to respiratory regulatory centres, including the brainstem, which can be affected by seizures [5, 8].

The amygdala is formed of six subnuclei with extensive interconnections [9, 10]. It is enriched in inhibitory, neuropeptidergic neurones and axonal networks [11-13] that modulate neuronal function [14-17] and are themselves modulated or augmented by chronic seizures [18, 19]. Following their release from dense core vesicles in CNS synapse it may be several hours before neuropeptide cellular stores are replenished [20] and transient depletion in the post-ictal period has been shown experimentally [21].

We recently observed reduced somatostatin and galanin in the ventrolateral medulla in a post mortem SUDEP series [22] and our aim was to carry out an investigation of neuropeptides in the amygdala. Through including a group of SUDEP cases with a

history of one or more seizures followed by recovery in the 24 hour period prior to the SUDEP, we aimed to address whether the occurrence of recent seizures also had any impact on amygdala neuropeptide levels.

Methods

Case selection

Amygdala tissue was obtained from 37 post mortem cases from the Epilepsy Society Brain and Tissue Bank at UCL, through Brain UK (pathology department at Derriford Hospital, Plymouth) and the MRC sudden death brain bank in Edinburgh. Tissue from all cases was retained with era-appropriate consent and the project has ethical approval (through NRES 17/SC/0573) and included three cause of death groups: 15 SUDEP cases, 12 epilepsy controls (epilepsy with non-SUDEP cause of death) and 10 non-epilepsy controls. Within the SUDEP group, 7 cases were selected where there was a history of a recent seizure (or cluster of seizures) with recovery, witnessed or reported during the 24 hour prior to the death (SUDEP-R); in the remaining 8 SUDEP cases this history was lacking (SUDEP-N). Only one of the SUDEP deaths was witnessed. The cases were acquired from post mortem examinations conducted between 1996 to 2015 and the post mortem intervals (time between death and post mortem) and tissue fixation times were recorded. The clinical details of the groups are summarised in Table 1 (further detail, including recent seizure history, anti-epilepsy drug medications, circumstances and cause of death, is included in supplemental Table 1).

The original formalin-fixed paraffin-embedded tissue blocks taken from the amygdala were retrieved from each case; in 18 cases, samples from both left and right amygdala were available (Table 1). Sections were cut at 5 micron thickness and one section was

stained with LFB/CV for anatomical orientation and delineation of the main human amygdala subnuclei, as previously described [23]. Immunohistochemistry for NPY (Sigma polyclonal 057K4869; 1: 5000), SST (Santa Cruz sc-7819, polyclonal; 1: 500), Galanin (Santa Cruz Monoclonal, sc-166431; 1: 5000) and synaptophysin (Dako monoclonal M7315 ; 1 : 100) was carried out on further sections using standard methods (see supplemental file for detail).

Quantitative analysis

The anatomical level of the amygdala was categorised as one of three coronal levels in a rostro-caudal direction: rostral amygdala (anterior to lateral ventricle), mid amygdala (at level of temporal horn) and caudal amygdala (level of anterior hippocampus). Slides were scanned with a Leica SCN400F digital slide scanner (Leica Microsystems, Wetzlar, Germany) at 40x magnification and were analysed with Definiens Tissue Studio software 3.6 (Definiens AG, Munich, Germany). Four amygdala subnuclei were outlined on the LFB/CV section: lateral, basal, accessory basal and the peri-amygdala cortex (PAC) (Figure 1a). The central nucleus is not represented in all caudal levels of amygdala and therefore not included in the analysis. Nuclei were only outlined if represented in entirety on the section (detailed in Table 1). ROI outlined on LFB/CV were then overlaid onto each of the immunostained sections to ensured similar ROI capture across cases and sections (Supplemental Figure 1A-D). Each section was carefully manually edited for any slight misalignment of ROI. An intensity threshold was set for the chromogen detection (Supplemental Figure 1E-G) and applied across all cases at x 40; the total labelling index (LI) (percentage area stained by cells and axonal processes) for the each ROI was measured.

Statistical analysis was carried out using SPSS version 22 (IBM corporation, CA, USA) using Mann-Whitney, Kruskall Wallis tests (for comparison between cause of death groups), Friedman test (for comparison of labelling within nuclei) and Spearman's test for clinical-pathology correlations; p values<0.05 were taken as significant. For graphical representation of data, Graphpad Prism 7 (University of California, San Diego) was used.

Results

Galanin immunohistochemistry showed dense axonal and dendritic networks in the amygdala subnuclei in all groups (Figure 1B, F, J, N) including scattered labelled neurones (Figure 1J). SST (Figure 1C, G, K&O) and NPY (Figure 1D, H, L&P) showed more frequent medium to large neurons (Figure 1C,G,&H) in addition to a more variable axonal networks. Quantitative analysis confirmed higher LI for Galanin than SST, with lowest values for NPY. Significant regional variations between lateral, basal, accessory basal and PAC ROI were noted over all cases ($p<0.01$ to $p<0.000001$) (Table 2) with highest levels for Galanin in the lateral nucleus, for SST in the accessory basal and NPY in the PAC in the control group.

Differences between cause of death groups

Galanin LI were significantly lower in SUDEP than epilepsy controls in all four amygdala regions ($p<0.05$); they were also lower in the lateral nucleus compared to non-epilepsy controls ($p<0.05$) (Figure 2A). SST LI were significantly lower in the SUDEP group than epilepsy controls in all four amygdala regions ($p<0.05$ to $p<0.005$) but not significantly lower compared to non-epilepsy controls (Figure 2B). NPY mean

LI were significantly lower in SUDEP than epilepsy controls in all four amygdala regions ($p<0.05$ to <0.0005) but were not significantly lower compared to non-epilepsy controls (Figure 2C).

There were no significant differences in Galanin, SST or NPY LI for any ROI between the SUDEP-R and SUDEP-N groups (Figure 2A-C, Table 2). Epilepsy controls showed higher LI for all markers compared to non-epilepsy controls (Figure 2A-C), significant for NPY LI in lateral and basal nuclei ($p<0.005$ and $p<0.05$). There was a significant positive correlation between LI for Galanin, NPY and SST for each ROI (Supplementary Figure 2). There were no significant differences in synaptophysin LI between any cause of death groups for any amygdala region (Figure 2D, Table 2).

Clinical and Pathology correlations

There were significant differences in the age of death between the three cause of death groups with older ages in the epilepsy-control group (Table 1). However there was no correlation between age of death and LI for Galanin, SST and NPY in any cause of death group. There were no significant differences in LI in relation to gender or for the presence of identified focal pathology (including a presumptive epileptogenic lesion). Full information available regarding age of onset, duration of epilepsy and seizure type and was not available in all cases and this was not further analysed; in addition a wide variety of anti-epilepsy drug medications was documented in both SUDEP and epilepsy controls which was not further evaluated (Supplemental Table 1).

LI in left versus right amygdala were not different in this series. Regarding the three coronal levels of the amygdala, the majority of samples analysed were from the rostral amygdala with no significant differences in levels included between the three cause of death groups (Table 1). There was also no significant variation in LI in subnuclei between the three coronal levels for any markers in the non-epilepsy controls. In SUDEP cases, significantly lower NPY LI was noted in basal, accessory basal and PAC in rostral levels and in epilepsy controls, lower SST LI in basal and PAC ROI at mid amygdala levels ($p<0.05$).

There were significant differences in fixation times and post mortem intervals (PMI) across the cause of death groups with longest PMI in epilepsy controls and longest fixation time in SUDEP-R group (Table 1). Tests on tissues fixed for 1 day to 5 months showed no qualitative reduction in labelling for SST, Galanin or NPY with longer fixation time (Supplemental Figure 1H-V). Over all cases there was also no significant reduction in LI with longer fixation times or PMI for all markers.

Discussion

In a post mortem series we have shown a global reduction in three neuropeptides in amygdala subnuclei in SUDEP compared to epilepsy controls and also compared to non-epilepsy controls for galanin in the lateral nucleus. We did not observe differences in neuropeptide labelling in SUDEP cases with documented seizures in the 24 hour period prior to death compared to those without this history. Clinical and imaging studies implicate amygdala involvement in ictal autonomic dysfunction, in particular apnoea, and vulnerability to SUDEP [3, 4, 8]. The identification of diminished

neuropeptides in SUDEP could be mechanistically relevant to peri-ictal amygdala dysfunction and autonomic phenomena.

Previous studies of amygdala neuropathology in temporal lobe epilepsy (TLE) have reported neuronal loss, more commonly involving the lateral and, to lesser extent, the basal nucleus with volume reduction [9, 24]. Alteration in dendritic spines and number of dendrites in TLE in the lateral, basal and accessory basal nuclei was also observed [25]. In the context of SUDEP, in a study of 15 cases significant neuronal loss and gliosis was shown in the lateral nucleus compared to non-epilepsy controls, but not in the central or basal nuclei [26]. In a further study of 28 SUDEP cases however, no difference in the quantitative analysis of GFAP in the lateral, basal and accessory basal nucleus was shown in SUDEP compared to an epilepsy control group, suggesting that there is no specific or signature pattern of damage [27]. In the present series, although two cases showed amygdala gliosis (supplemental Table 2), there was no clear evidence of neuronal loss or amygdala sclerosis in epilepsy cases, supported by quantitative synaptophysin labelling which was not different between the three cause of death groups. Furthermore, MRI studies in SUDEP have reported increased grey matter volumes in the right amygdala [4] although it is acknowledged that the pathological correlate for MRI volume changes remains to be explored [3].

Diminished amygdala neuropeptides in SUDEP

The overall lower levels of amygdala neuropeptides in SUDEP compared to normal controls were significant for galanin. The amygdala has a diverse range of neuropeptides that modulate neuronal and synaptic activity [14-17]. Stored in large dense vesicles in interneurons and released on high frequency firing, many neuropeptides have reputed anti-seizure effects [21], exerting both pre- and postsynaptic actions on

local excitatory and inhibitory transmission [20, 28]. The human amygdala is known to be relatively enriched in galanin [13, 29] and has one of the highest levels of NPY [11] and SST [12] compared to other brain regions. As neuropeptides are degraded by peptidases and not immediately replenished for several hours following release [20], one explanation for diminished levels in SUDEP is their cellular consumption or 'exhaustion' following a seizure with resultant neuronal fatigue. Depletion of neuropeptides has been shown following experimental seizures or status epilepticus, for example depletion of galanin was observed in the dentate gyrus following 30 minutes of seizures [21].

Recent poor seizure control is often documented in SUDEP, which could further impact on neuropeptide neuronal reserves. For example in the MORTEMUS study of monitored SUDEP, several patients had two or more generalised seizures the day before death [2]. We were able to select SUDEP cases from our archive where there was clear documentation of one or more seizures, followed by recovery, in the immediate 24 hours prior to the SUDEP. These cases, however, did not show any significant neuropeptide reduction compared to SUDEP cases without this documented history. We acknowledge there may be limitations regarding the clinical data, in that not all seizures occurring in the 24 hours prior to death may have been documented or witnessed. Furthermore, SUDEP is likely to be a post-ictal event even in unwitnessed cases, as were the majority in this study; it is remains plausible that this final fatal seizure itself mediates significant or catastrophic neuropeptide release and consumption. We also noted a correlation between the labelling for galanin, SST and NPY suggesting synchronous activity and release of neuropeptidergic neurones. In a recent study of the brainstem using a different SUDEP post mortem cohort, we also observed reduction of SST and galanin in the ventro-lateral medulla respiratory nuclei [22], supporting the view that concurrent neuropeptide depletion could occur in

several critical brain regions for autonomic and respiratory regulation. It is conceivable that in the acute 'post-ictal' amygdala, pivotal neuropeptide depletion results in decompensation of networks that normally modulate respiration following a seizure.

Enhanced amygdala neuropeptide networks in non-SUDEP epilepsy cases

We also identified higher amygdala neuropeptide labelling in epilepsy controls than normal controls, reaching significance for NPY, in support of compensatory augmentation as a result of repetitive seizures. The marked plasticity of neuropeptidergic neurons and their receptors has been previously shown in both experimental seizure models and human studies, supporting the innate adaptability of these systems [14-16, 21, 30]. Galanin is regarded as one of the most inducible neuropeptides [21] and increased mRNA expression in the amygdala has been shown following experimental seizures [31]. Increased expression and axonal sprouting of NPY and SST axons is observed in TLE [30, 32, 33] as well as in seizure models, supporting their plasticity, particularly prominent in the limbic system [18, 19]. For example, in an experimental kindling model, a significant increase in amygdala SST was observed after 2 months following seizure onset [34]. In the kainic acid model, NPY-labelled fibres were increased in the amygdala [18]. The method we used enabled detection of both somal and axonal-dendritic amygdala networks over the entire nucleus area to evaluate any axonal-dendritic sprouting. A further key observation was the significantly lower levels of all neuropeptides in SUDEP compared to the epilepsy control group. This raises a further concept that in SUDEP there is failure of these 'normal' adaptive and presumed anti-epileptogenic neuropeptide alterations, acting as a further vulnerability factor.

Regional function of the amygdala and relevance to SUDEP

Histological studies demonstrate that the amygdala subnuclei and PAC, apart from central nucleus, are reciprocally interconnected [9, 35 36]. Functional connectivity maps of the human amygdala confirm regional amygdala differences that correlate with histologically defined subnuclei; the lateral-basal nuclear groups show widespread cortical connections and the centromedial groups project to regions with putative roles in arousal and autonomic regulation [37], including brainstem reciprocal connections [10]. The lateral nucleus can generate spontaneous interictal activity [38] and ictal central apnoea has been reported with seizure spread to the amygdala [5]. In a recent study of the amygdala kindling model of epilepsy, amygdala stimulation was associated with reduced ictal ventilation and c-fos expression in neurones in brainstem respiratory nuclei [39]. Clinical studies have demonstrated apnoea following electrode stimulation of the amygdala in people with epilepsy under investigation [6-8] ; apnoea occurred following stimulation of the medial amygdala in one study [6] but following stimulation of lateral and basolateral nuclei in an earlier study but without lateralisation [8]. MRI series have shown left-right amygdala volume asymmetries in SUDEP [4]. We did not observe any left to right differences for our measures, but there was evidence for greater alteration in the caudal amygdala and more significant changes in the lateral nucleus in SUDEP. This may reflect regional differences in neuropeptide distribution between subnuclei of potential functional relevance. For example SST mRNA was shown to be lower in lateral than basal nucleus [40], in keeping with current quantitative analysis in the present study. Any regional vulnerability in the amygdala in SUDEP requires further investigation and how it functionally relates to interconnected regions in the extended amygdala and beyond.

Limitations of the study

This post mortem study utilised archival material and the central nucleus, which is relevant to autonomic amygdala functions, was represented in only a proportion of the cases and not analysed. Conventional anti-epilepsy drug treatments varied across the cases; although not known to directly act on neuropeptidergic systems, the exact mechanism of action of some drugs is unclear and we cannot exclude secondary effects. Variations in fixation times and variable post mortem intervals were noted between the cause of death groups. However the longest interval occurred in epilepsy controls which showed the highest neuropeptide labelling index. Further studies are required, including proteomics, neuropeptide receptor analysis and gene expression studies to corroborate current immunohistochemistry findings. It is also important to study larger, stratified SUDEP series to further explore the potential role of neuropeptides in SUDEP.

In summary, significant reduction in galanin in the lateral nucleus in SUDEP could represent acute, transient depletion following seizures of relevance to post-ictal amygdala dysfunction and apnoea. Increased levels of amygdala neuropeptides noted in epilepsy controls is likely to be seizure-induced adaptive augmentation ; this appears to be deficient in SUDEP cases and could represent a further vulnerability factor for amygdala dysfunction in the post-ictal period.

KEY POINTS BOX

- Clinical and experimental studies implicate amygdala dysfunction in peri-ictal apnoea associated with SUDEP.

- In a post mortem series reduced galanin in the lateral nucleus compared to non-epilepsy controls was shown which could impair normal amygdala autonomic functions.
- Reduced SST, galanin and NPY in amygdala subnuclei in SUDEP compared to epilepsy controls could indicated deficient adaptive alterations in response to seizures.

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STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The study has ethical approval with appropriate consent for research for all cases included in the study. All data generated or analysed during this study are included in this published article. None of the authors have conflicts of interest to declare.

FIGURE LEGENDS

Figure 1. Regions of interest and neuropeptide immunohistochemistry in lateral nucleus of amygdala

A. The four regions of interest (ROI, lateral , basal, accessory basal and peri-amygdala cortex (PAC)) were defined on a Luxol fast blue/ Cresyl violet (LFB/CV) stained section and outlined at x 40 (shown here at low power) ; these ROI were then superimposed on the immunostained sections for synaptophysin , galanin, SST and NPY (shown in supplemental Figure 1A-D) in SUDEP cases with a history of seizures in the 24 hour period prior to death (SUDEP-R) **(B-D),** SUDEP with no history of recent seizure (SUDEP-N) **(E-H),** epilepsy controls (EPC) (**I-L**) and non-epilepsy controls (NEC) **(M-P).** Synaptic or variably dense axonal-dendritic networks were present with scattered positive cells (arrows). All regions shown are for the lateral nucleus and the neuropeptide labelling index for the whole ROI is as follows in these illustrated cases: B=8.9%, C=6%, D= 0.7%, F=16% G=4%, H=2.7%, J=92%, K=53%, L=89%, N=74%, O=76%, P= 73%. Bar B to P = 100 microns

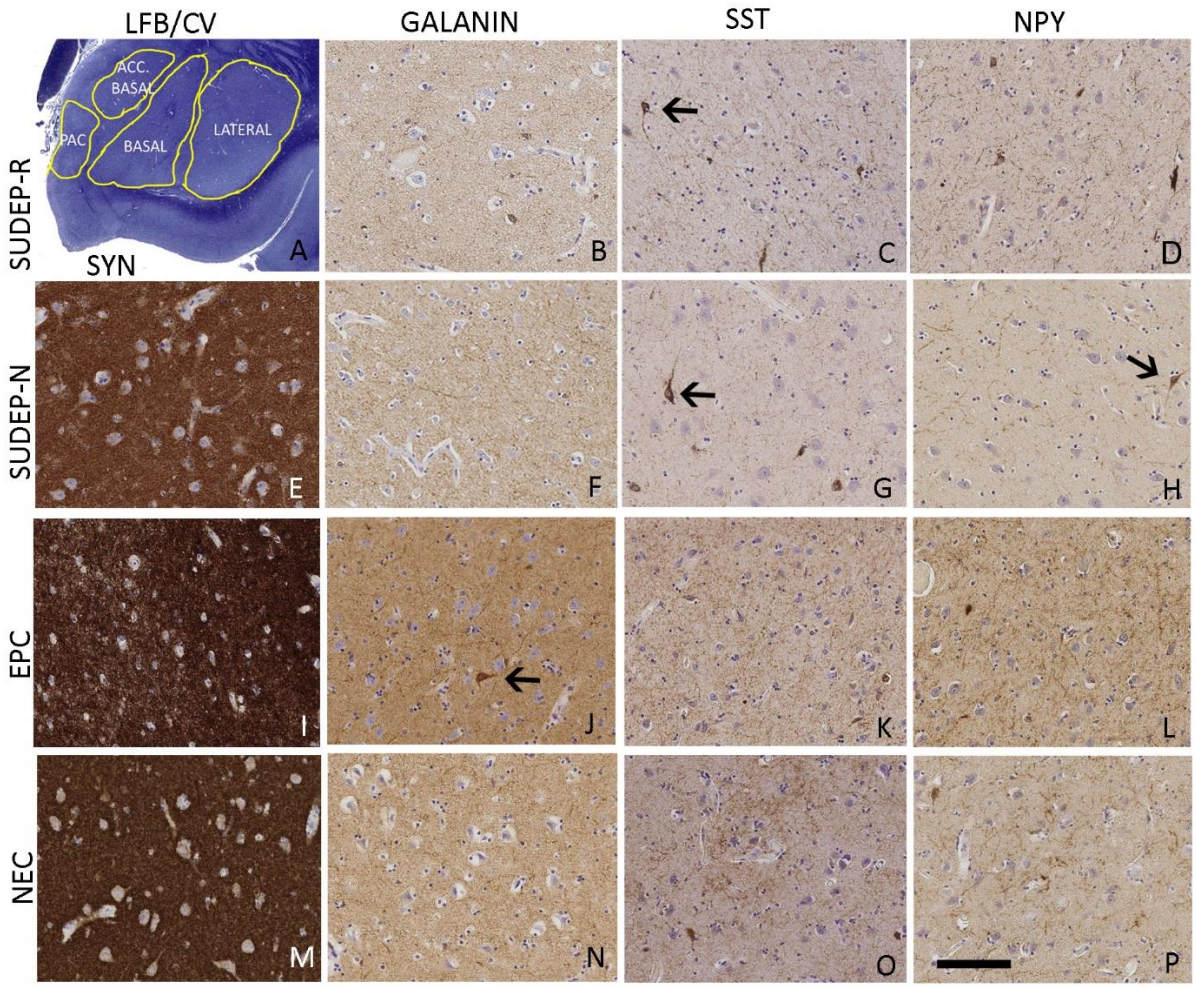
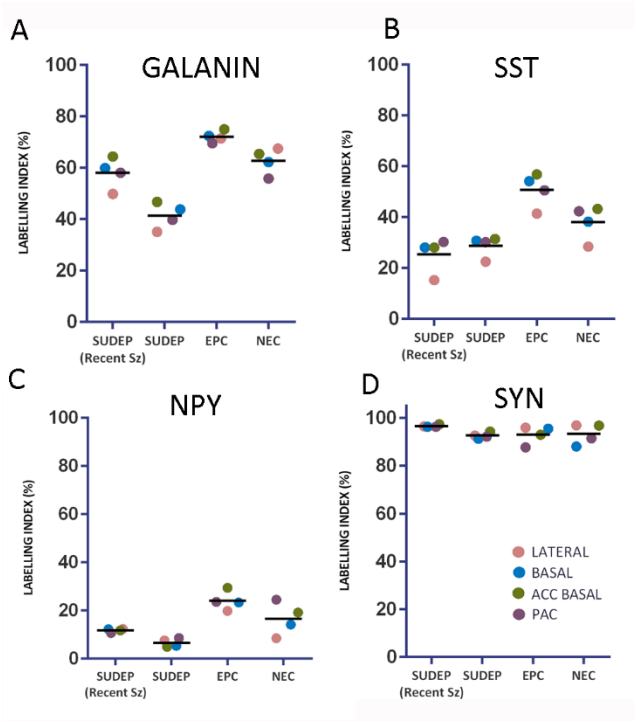


Figure 2. Scatter plots of mean labelling index of cases in cause of death groups. **A**. Galanin, **B**. Somatostatin (SST), **C.** Neuropeptide Y (NPY) and **D.** synaptophysin. The SUDEP group was divided into SUDEP (Recent Sz) cases with a history of seizures in the 24 hour period prior to death and other SUDEP with no history of recent seizure. Standard deviations of the data is provided in Table 2.

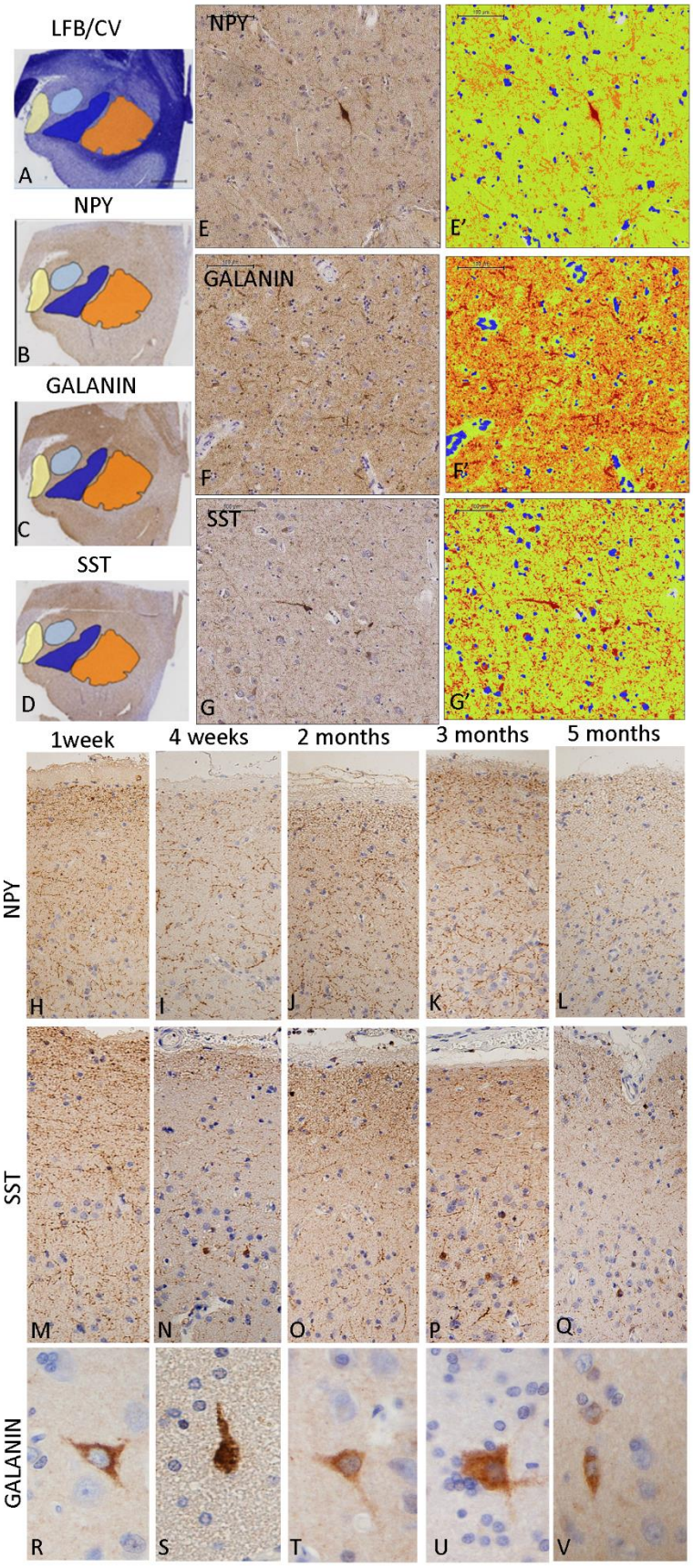


Supplemental Figure 1. Image analysis methods and effects of formalin fixation times on immunohistochemistry

A-D: Example of regions of interest (ROI) drawn on LFB/CV (A) section and transposed to adjacent NPY (B), galanin (C) and SST (D) stained serial sections ; ROI shown as lateral (orange), basal (dark blue), accessory basal (light blue) and peri-amygdala cortex (yellow).

E, F &G: Examples of Definiens image analysis system for thresholding of labelling index and detection of cell bodies and extensive axonal and dendritic processes. Red shows high intensity, orange mid intensity, yellow low intensity and blue for haematoxylin. For the data analysis all high and mid intensity labelling was included as positive. **E**. NPY, **F**. Galanin and **G**. SST with threshold images shown in **E', F'** and **G'.**

H-V: Fixation time series of a surgical temporal lobe fixed between 1 week and 5 months in 10% formalin solution prior to tissue processing in order to address the effects of fixation on immunohistochemistry for NPY (top row), SST (middle row) and galanin (bottom row). There was no qualitative diminution in labelling over 3 months period and the mean fixation times in all the post mortem cause of death groups was less than 44 days (Table 2).


LFB/CV
A
NPY
B
GALANIN
C
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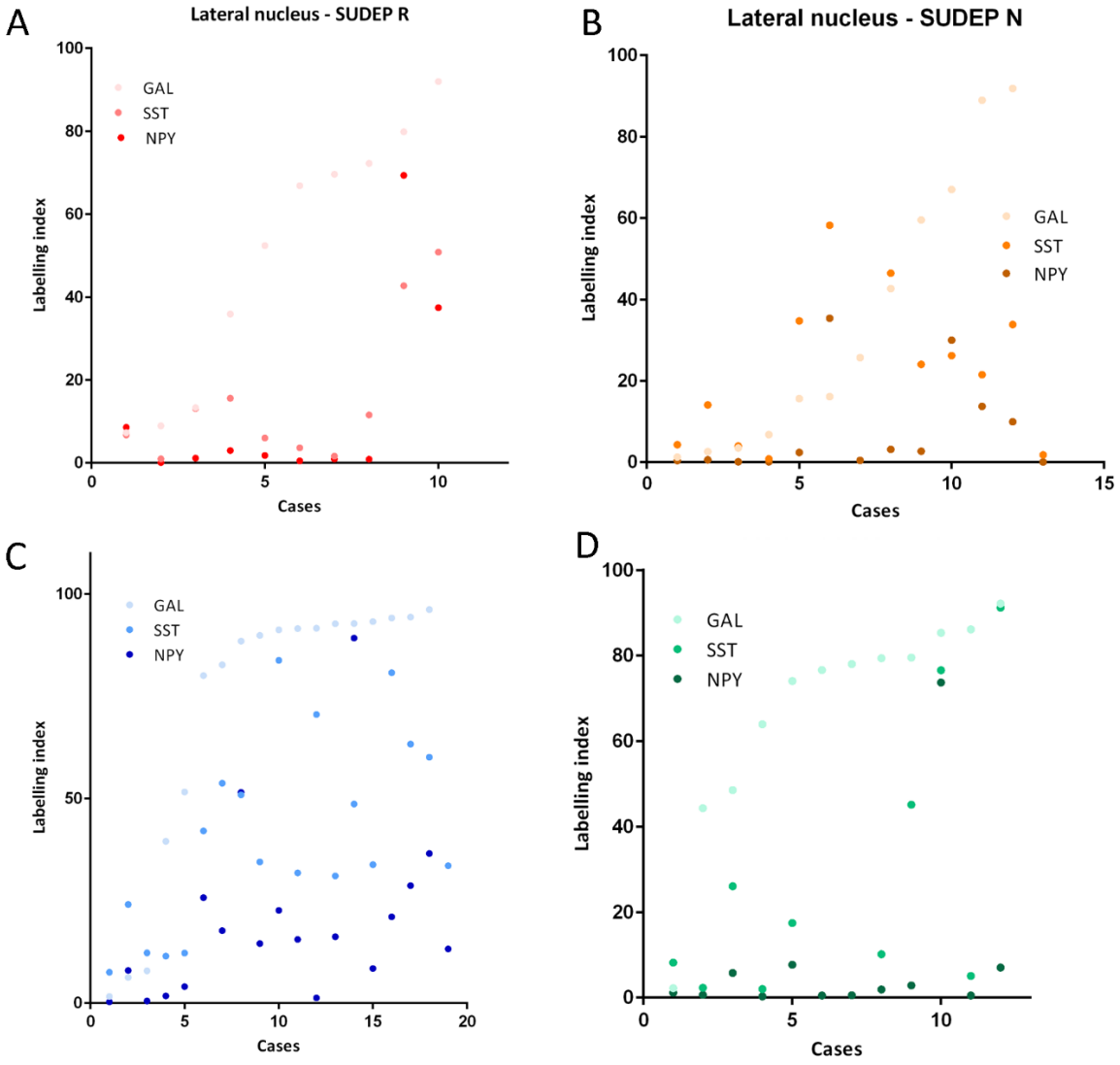
Supplemental Figure 2 : Scatter graphs of labelling index for galanin, NPY and SST for individual cases sections in each cause of death group.

The data is illustrated for each section (left and right) in the lateral nucleus only for cause of death groups with increased galanin on the x axis of the graph. There were lower levels of NPY and SST labelling compared to galanin, but an overall correlation between NPY, SST and galanin labelling index was noted**. A.** SUDEP R (SUDEP with recent seizures in 24 hours prior to death), **B**. SUDEP (no history of recent seizures), **C**. EPC (epilepsy controls), **D**. NEC (non-epilepsy controls).



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