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Review: Neuropathology findings in autonomic brain regions in SUDEP and future research directions

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Abstract

Autonomic dysfunction is implicated from clinical, neuroimaging and experimental studies in sudden and unexpected death in epilepsy (SUDEP). Neuropathological analysis in SUDEP series enable exploration of acquired, seizure-related cellular adaptations in autonomic and brainstem autonomic centers of relevance to dysfunction in the peri-ictal period. Alterations in SUDEP compared to control groups have been identified in the ventrolateral medulla, amygdala, hippocampus and central autonomic regions. These involve neuropeptidergic, serotonergic and adenosine systems, as well as specific regional astroglial and microglial populations, as potential neuronal modulators, orchestrating autonomic dysfunction. Future research studies need to extend to clinically and genetically characterised epilepsies, to explore if common or distinct pathways of autonomic dysfunction mediate SUDEP. The ultimate objective of SUDEP research is the identification of disease biomarkers for at risk patients, to improve post-mortem recognition and disease categorisation, but ultimately, for exposing potential treatment targets of pharmacologically modifiable and reversible cellular alterations.

Keywords

SUDEP; Brainstem; respiratory nuclei; amygdala; neuropathology

1. Introduction

In the last decade there have been considerable advances made in our understanding of sudden and unexpected death in epilepsy (SUDEP), from neurogenetics (Goldman et al., 2016), functional and structural neuroimaging (Allen et al., 2019a), clinical physiological studies (Lhatoo et al., 2015; Ryvlin et al., 2013), and experimental modelling (Noebels Jl Md, 2019). This has been accompanied by better recognition that this is the leading cause of

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epilepsy-related death in young adults with epilepsy (Devinsky et al., 2016; Middleton et al., 2018). Through ongoing cross-disciplinary studies, disease mechanisms can be interrogated, and risk-factors and disease biomarkers discovered with the future goal of implementing prevention strategies. Neuropathology can contribute to this process. Although examination of the brain in SUDEP may appear initially unremarkable, systematic analysis of large series has the potential to shed light on common patterns of seizure-related cellular alterations that may have reduced the brain's resilience to the physiological challenges of seizures.

SUDEP has been defined as an unexpected and non-accidental death in patients with epilepsy (excluding status epilepticus), where no cause of death is identified following post-mortem examination including neuropathology (Nashef et al., 2012). The worldwide incidence is 1 to 2 per 1000 people with epilepsy per year (Harden et al., 2017; Sveinsson et al., 2017; Thurman et al., 2014). SUDEP affects all age groups, but primarily young, otherwise healthy adults, peaking at 20–40 years and in epilepsy with diverse causes (Devinsky et al., 2016). Deaths mainly occur in the peri-ictal period, although most are nocturnal and unwitnessed. Although the mechanisms and pathophysiology are still uncertain, there is accumulating evidence from clinical, imaging and experimental studies to indicate a failure of central autonomic regulation and, in particular, of respiratory dysfunction (Devinsky et al., 2016; Jefferys et al., 2019; Ryvlin et al., 2013).

The focus of this review is on recent neuropathological findings in the investigations of respiratory and autonomic brain regions in SUDEP, in the context of the emerging clinical and experimental data, and future directions for research.

2. Clinical, evidence for autonomic dysfunction in seizures and SUDEP

The primary risk factor, based on meta-analysis of SUDEP series, is poor control of generalised tonic-clonic seizures (DeGiorgio et al., 2017; Harden et al., 2017). During and following seizures autonomic phenomena have long been reported including prolonged periods of irregular, shallow breathing (Simon, 2010). Patients with documented central apnoea with associated hypoxaemia occurring around the time of a seizure (the peri-ictal period) are regarded at greater risk for SUDEP (Bruno et al., 2018; Lacuey et al., 2018; Vilella et al., 2018). Sympathetic outflow dominates after a seizure ('sympathetic storm') with effects on blood pressure, heart rate (Hampel et al., 2016) and even baroreflex sensitivity (Esmaeili et al., 2018; Hampel et al., 2017). Simultaneous parasympathetic and sympathetic stimulation or autonomic dysynchrony has also been proposed as a SUDEP risk factor, as reflected by an abnormal heart rate variability in some epilepsy patients' (DeGiorgio et al., 2010; Page et al., 2018). As SUDEP often occurs nocturnally or during sleep (Sveinsson et al., 2018) the convergence of brainstem arousal systems interacting with respiratory control during seizures may be an additional critical factor. Most SUDEP deaths are unwitnessed but documentation of the rare seizure deaths occurring while patients are on epilepsy monitoring units, where anti-seizure medications are withdrawn under video, EEG and cardio-respiratory monitoring, have provided valuable insight. In the MORTEMUS study (Ryvlin et al., 2013), SUDEP followed a generalized seizure with depressed arousal and respiration, non-tachyarrhythmic cardiac dysfunction, profound depression of cortical EEG activity with terminal apnoea within 11 mins of the seizure and preceding terminal

asystole (Ryvlin et al., 2013). This was interpreted as early, centrally mediated, respiratory and cardiac dysfunction, also referred to as a 'postictal neurovegetative breakdown'.

3. Insights from post-mortem neuropathology

Neuropathologists performing autopsies have recognised for centuries that patients with epilepsy can die suddenly with no anatomical or toxicological cause of death found, i.e. a 'negative' post-mortem examination (Krohn, 1963; Sommer, 1880). In studies of post-mortem SUDEP series, common neuropathology findings include mild degrees of cerebral oedema (gyral effacement, uncal grooving) and congestion; in a larger series, macroscopic lesions were identified in 52% (old traumatic lesions 17%, hippocampal sclerosis HS 21%, cortical malformations 15%) (Thom et al., 2016). From the published series to date however (Thom et al., 2018), there is no evidence that any specific lesional pathology carries a greater burden of risk for SUDEP. It is also recognized that epilepsy can induce widespread cellular changes, recognized in the post-mortem brain, likely representing secondary or 'acquired' neuropathology. These alterations encompass diverse processes from neuronal or synaptic loss, neuronal hypertrophy, axonal sprouting to glial and inflammatory activation (Blanc et al., 2011; Sinjab et al., 2013; Thom et al., 2012; Thom et al., 2005). One hypothesis proposes that repeated generalised seizures leads to accumulative brain changes and increased susceptibility to SUDEP (Figure 1).

Neuropeptidergic systems, for example, show significant dysregulation in epilepsy (Mazarati, 2004) and represent molecular candidates for exploration in SUDEP for several reasons. Neuropeptide Y, galanin, somatostatin, substance P, and dynorphin have widespread CNS distribution, particular in autonomic and brainstem centres, and are powerful modulators of neurotransmitter activity (Chi et al., 2018; Clynen et al., 2014; Kovac et al., 2013). Stored in large dense vesicles in interneurons and released on high frequency firing, they have a long half-life with long-lasting effects, interact with monoaminergic systems and exert both pre- and post-synaptic actions. Many also have anti-seizure effects (Mazarati, 2004) but are degraded by peptidases and can be transiently depleted following seizures or status epilepticus (Mazarati, 2004). Extensive experimental and human studies demonstrate alterations of neuropeptidergic neurones, neuropeptide release, axonal networks and neuropeptide receptors in limbic and cortical regions and the malleability of these systems in response to seizures and epilepsy (Chi et al., 2018; Clynen et al., 2014; Kovac et al., 2013; Mazarati, 2004; Thom, 2014).

Seizure-related neuropathology likely reflects the direct effects of cell injury (reversible or irreversible) and adaptive or re-organisational plasticity, possibly as neuroprotective phenomena, but which can further modulate neuronal function and both potentiate or dampen pro-epileptogenic networks. If such plasticity involves CNS autonomic regulatory regions there is a theoretical vulnerability for defective or inappropriate autonomic responses during seizures and therefore to SUDEP (Figure 1). In brainstem respiratory networks there is a recognized, remarkable capacity for compensatory adaptation or 'fine tuning' to respond to physiological challenges, including intermittent or sustained episodes of increased pCO_2 or reduced O_2 (Clayson et al., 2020; Dereli et al., 2019; Doi et al., 2010; Kang et al., 2017; Mitchell et al., 2001; Reeves et al., 2006; Smith et al., 2013). For example,

a reduction in medullary serotonergic neurones occurs in an experimental conditions of induced hypercapnia (Burgraff et al., 2019). Such 'respiratory neuroplasticity' may conceivably occur in patients in response to frequent generalized seizures and associated ictal hypoxaemias (Lacuey et al., 2018). Indeed, in a clinical study of epilepsy patents on monitoring units, reduced ventilatory responses to increased pCO_2 was observed in some patients and associated with the severity of postictal CO_2 levels as well as SUDEP (Sainju et al., 2019). Postictal hypoperfusion of brainstem respiratory centres was observed in all patients following generalised but not focal seizures (Liu et al., 2020) and a neuroimaging study noted more severe brainstem volume loss in epilepsy patients with severe ictal hypoxia (Allen et al., 2020). Furthermore, brainstem volume reduction on MRI in TLE correlated with reduced heart rate variability (Mueller et al., 2018). In summary, brainstem pathology may be a consequence of seizures but in turn can mediate autonomic dysfunction in subsequent seizures.

4. Neuropathology studies of brain stem autonomic centres in SUDEP.

4. 1 Pre-Bötzinger complex

Brainstem respiratory rhythm generating circuitry comprises interacting nuclear groups in the medulla and pons, the Bötzinger and pre-Bötzinger complex (pre-BötC) and retrotrapezoid nucleus (RTN), under modulation by interconnecting nuclei (Smith et al., 2013) (Figure 2). The pre-Bötzinger complex is the principal kernel of inspiratory rhythm generation with pacemaker activity (reviewed in (Ghali, 2019)), multi-transmitter systems, complex interconnections and high modulation to accommodate adaptation under normal physiological conditions (Ramirez et al., 2012). Although extensively studied in animals, its anatomical location in the human medulla was only outlined in 2011 (Schwarzacher et al., 2011). It is composed of pacemaker-like somatostatin and excitatory neurokinin 1 receptor (NK1R) positive cells (Guyenet et al., 2001) as well as glycinergic and GABAergic interneurons; these form an ill-defined region in the reticular zone of the ventro-lateral human medulla (VLM) extending from obex level 6mm to 12mm. In animals there a rostro-caudal organisation of the ventral respiratory groups in the VLM; the Bötzinger complex (expiratory rhythms) and pre-BötC are more rostral than ventral respiratory groups that co-ordinate output to the phrenic and spinal motor neurones (Smith et al., 2013).

In-vivo quantitative MRI studies in SUDEP have shown volume loss in the medulla (Mueller et al., 2014; Mueller et al., 2018) although not in all studies (Allen et al., 2019b). This was recently further investigated in post-mortem brainstem samples using high field 9.4 Tesla MRI (Figure 3a,b) in addition to the Cavalieri stereological method on tissue sections for regional medullary volume estimations. Defined autonomic regions of the reticular zone and the VLM in SUDEP groups were compared to controls (Patodia et al., 2020b). Controls included non-epilepsy sudden deaths and epilepsy controls without a SUDEP. With these two methods, decreased volumes, specifically in the VLM region (but not in other brainstem regions) were identified in the rostral medulla in epilepsy compared to non-epilepsy cases, with evidence for greater volume reduction in SUDEP. In contrast, in the caudal medulla greater reticular zone volumes were observed in the SUDEP group. Our observation of lower

volumes in SUDEP cases in the rostral medulla could be relevant to acquired pathology localising with the pre-BötC region.

In parallel studies neuronal subtypes in the VLM were quantified. A reduction in somatostatin neuronal labelling in the VLM, most significantly in the rostral medulla (obex 7–9mm) was found in the SUDEP group compared to normal controls (Patodia et al., 2018), therefore aligning with the previously defined level of the pre-BötC (Schwarzacher et al., 2011) (Figure 2 and 3). Somatostatin is a neuropeptide which acts as an inhibitory respiratory modulator (Cui et al., 2016; Kaczynska et al., 2018) with endogenous release in the pre-BötC stabilising breathing rhythmicity during normoxia (Kaczynska et al., 2018). Subgrouping somatostatin neurones, a specific reduction in cells with a peripheral rim of labelling was observed, in keeping with synaptic terminals, but not in neurones with diffuse cytoplasmic labelling, interpreted as the endogenously somatostatin synthesising pre-BötC neurones (Figure 4d–f). Animal studies show that somatostatin projections to the pre-BötC neurones arise mainly from the parabrachial nucleus, solitary tract nucleus as well as other regions (Bou Farah et al., 2016), modulating respiratory activity (Cui et al., 2016; Epelbaum et al., 1994) (Figure 2). Our findings therefore supported a reduction in modulating input, rather than a reduction of pre-BötC somatostatin synthesising capacity in SUDEP. Neurokinin 1 receptor (NK1R) expression (Patodia et al., 2018), present in different regions of the ventral respiratory complex (Schwarzacher et al., 2011; Wei et al., 2012) was also investigated as a second pre-BötC neuronal marker, co-localising with somatostatin neurones (Patodia et al., 2018). NK1R is preferentially activated by the neurokinin substance P, which has functionally diverse roles in respiratory reflexes (Szereda-Przestaszewska et al., 2020). Quantification of NK1R labelling in SUDEP (Figure 4b) showed a reduction in the caudal medulla VLM only, at obex level 3–4mm compared to control groups. Interestingly, stereological quantification of total VLM neurones with cresyl violet stain (Figure 4a) also identified a reduction in neuronal number at this obex level only (Patodia et al., 2018). These findings together with the MRI study of higher reticular zone volumes in SUDEP in the caudal medulla (Patodia et al., 2020b) implicate additional pathology in the ventral respiratory groups that determine the motor control of respiration (Figure 2, 3).

4.2 Serotonergic medullary networks in SUDEP

Serotonin (5-HT), synthesised in the medullary raphe nuclei, acts as an excitatory modulator of inspiration, stimulating respiratory centres, including the pre-BötC neurones in response to hypercapnia (Richerson, 2013; Szereda-Przestaszewska et al., 2020) and has been a focus of recent SUDEP research (Murugesan et al., 2018; Petrucci et al., 2021; Richerson, 2013; Richerson et al., 2016; Tupal et al., 2006; Uteshev et al., 2010; Zhan et al., 2016). Rostral brainstem serotonergic monoaminergic networks from the dorsal raphe also modulate arousal and myriad physiological brain functions through their widespread projection to other brain regions (Beliveau et al., 2017). Serum serotonin levels are raised following generalized but not focal seizures (Murugesan et al., 2018) and abnormal serotonergic neuronal firing during seizures has been observed experimentally (Zhan et al., 2016). 5-HT is known to inhibit seizures, reduce seizure susceptibility and 5-HT availability contributes to the anticonvulsant action of several common anti-epilepsy drugs (Petrucci et al., 2020). A recent study also showed a lower incidence of ictal central apnoea in patients receiving

serotonin reuptake inhibitors (SRIs) that act on the pre-synaptic serotonin transporter (SERT) (Lacuey et al., 2019), highlighting potential therapeutic applications.

The 5-HT synthesising neurones (tryptophan hydroxylase (TPH2) expressing) in the medulla are primarily located in the midline raphe nuclei with smaller numbers in the VLM region (Benarroch, 2014) (Figure 4g). There is extensive post-mortem literature implicating alterations of serotonergic neuronal populations in sudden infant death (Kinney et al., 2019), with fewer reports in adult studies of neurodegenerative conditions with autonomic dysfunction and sudden death (Presti et al., 2014; Schwarzacher et al., 2011; Tada et al., 2009). Quantifying TPH2 and SERT expressing neurones in the VLM and medullary raphe (Figure 4h,i) showed a reduction in SUDEP compared to controls groups (Patodia et al., 2018); specifically, this was noted for TPH2 expressing cells in the rostral VLM at obex level 7–9 mm and for SERT in the raphe nucleus at obex level 7–8mm (Figure 2,3). This suggests reduced 5-HT synthesising capacity in the VLM region as well as re-uptake by serotonergic neurones in the Raphe which may manifest as an overall reduction in availability in the pre-BötC region, of relevance in the vulnerable hypercapneic post-ictal period in terms of diminished reserves for augmenting ventilatory responses.

4. 3 Catecholaminergic medullary neurones

The above findings support alteration of specific neuronal groups in the medulla in SUDEP, more likely to be adaptive and potentially reversible. The question remains if alterations involve the brainstem systems more widely or if there is specific vulnerability of respiratory nuclear groups in epilepsy. The catecholaminergic neurones of the medulla (Tyrosine hydroxylase (TH) expressing, the rate-limiting enzyme in the synthesis of DOPA), include neurones within the VLM (C1 adrenergic neurones) that form the putative vasomotor centre regulating blood pressure but also influencing respiratory drive (Guyenet et al., 2013). VLM catecholaminergic neurones project to the pre-BötC, stimulating breathing in hypoxic conditions (Kang et al., 2017) and sleep-state dependent cardio-respiratory arousal (Abbott et al., 2013), of relevance to SUDEP as many deaths occur nocturnally. Experimental depletion of catecholaminergic neurones in the VLM impairs respiratory responses (Malheiros-Lima et al., 2017). Other groups of TH-expressing neurones are located in the dorsal medial medulla near the nucleus tractus solitarius, but their physiological function is less understood (Sevigny et al., 2012). Experimental studies have shown that both seizures (Silveira et al., 2000) and hypoxia (Kanter et al., 1996) increase VLM catecholaminergic neuronal activity which may underlie the increased blood pressure and sympathetic activity observed following some seizures (Simon, 2010).

Using similar cohorts (SUDEP, epilepsy-control groups and non-epilepsy controls) as in the study of Pre-BötC neurones, quantification of TH-expressing neurones in the VLM and dorsal medulla (Figure 4j) did not reveal any differences between cause of death groups. This contrasts to studies in Sudden infant deaths (SIDS), where a reduction of medullary TH-IR neurones was identified in some (Obonai et al., 1998; Ozawa et al., 2002) although not all series (Sawaguchi et al., 2003) and also in adults with Multiple System Atrophy associated with sudden death (Benarroch et al., 2005; Tada et al., 2009). Preservation of TH

neurones in SUDEP also contrasts with the reduction of TPH2 neurones in the VLM, in support of a selective vulnerability.

However, neuronal preservation does not exclude catecholaminergic neuronal dysfunction. C-fos, an early/immediate gene and marker of recent neuronal and synaptic activation has been used experimentally to identify acute seizure-related brain injury (Barros et al., 2015; Herrera et al., 1996; Mraovitch et al., 1999) as well as monitoring respiratory neuronal activation (Wang et al., 2015). c-fos in SUDEP appeared distributed primarily in medullary autonomic regions, including TH and TPH cells (Patodia et al., 2020c) but with fewer c-fos neurones in SUDEP compared to non-epilepsy sudden death controls. Although this could indicate diminished activation in VLM neurones prior to death, due to the limitations of post-mortem analysis and ante-mortem factors, other measures of neuronal activation are needed to validate this finding (Hudson, 2018; Sauvage et al., 2019) (Herrera et al., 1996).

Furthermore, in a case of severe childhood epilepsy following perinatal hypoxic-ischaemic brain injury and subsequent sudden death, intracellular polyglucosan bodies were observed and primarily located in the TH neurones of the medulla (Patodia et al., 2021). Polyglucosan bodies represent sequestered aggregates of ubiquitinated and p62 enriched insoluble glucose polymers and likely represent a cellular protective mechanism in response to a pathogenic insult (Brewer et al., 2020). Interestingly, a predilection for p62 inclusions in the dendrites of brainstem catecholaminergic neurones has been observed with aging, suggesting an intrinsic susceptibility of these neurones to cellular metabolic stresses (Braak et al., 2013). Accelerated ‘aging’ of TH medullary populations in epilepsy could conceivably reduce their functional capacity without neuronal loss which needs to be a future focus of research.

4.4 Galaninergic medullary networks

Galanin is a bioactive peptide, with overall inhibitory action, that modulates noradrenergic and serotonergic networks and the pre-BötC (Medel-Matus et al., 2017). Brainstem galaninergic neurones are primarily located in the nucleus of the solitary tract, VLM, retrotrapezoid nucleus (RTN) and locus coeruleus (Bochorishvili et al., 2012; Spirovski et al., 2012). The neurones of the RTN, critical for central respiratory chemoreflexes and responses to $pC0_2$, synapse with NK1R neurons of the pre-BotC and activate breathing (Bochorishvili et al., 2012). In SUDEP cases, a decrease in galanin immunolabelling in the VLM region, but not in the medullary raphe, was shown compared to control groups (Patodia et al., 2018) (Figure 4c). In rodents, there is no projection from the RTN galaninergic neurons to the medullary raphe (Bochorishvili et al., 2012) (Figure 2); these observations could therefore indicate dysfunction of the RTN nucleus in SUDEP. A recent experimental study, showed reduced galanin mRNA in the RTN following a short exposure of six hours to 5% pCO_2 yet with the opposite effect following long-term exposure (Dereli et al., 2019). In a paediatric study, depressed CSF galanin was noted in epileptic encephalopathy but elevated during periods of status epilepticus (Tekgul et al., 2020). Further in-depth investigation of the human galaninergic brainstem networks, its modulation by seizures or seizure-related apnoea and impact on SUDEP mechanism is needed.

4. 5 Medullary glia in SUDEP

Specific subsets of glial cells in the brain stem also act as chemoreceptors to $pC0_2$ levels, modulating neuronal function via gliotransmitters, such as adenosine (Falquetto et al., 2018) through connexin hemichannels (Huckstepp et al., 2010). They have been identified on the medullary surface (Huckstepp et al., 2010; Sobrinho et al., 2017), in the RTN (Sobrinho et al., 2017) and in the pre-BötC (Ikeda et al., 2017) and considered an integral component of respiratory homeostasis systems (Czeisler et al., 2019), including during physiological challenges (Sheikhbahaei et al., 2018b). Reduction in these specialised glia has been associated with respiratory dysfunction experimentally (Fernandes-Junior et al., 2018). In SUDEP post-mortem cases, lower densities of regionally specific and morphologically distinct glial cell types, labelled with vimentin, connexin43 and adenosine receptor A1R (Figure 4k,l), were observed in the VLM, along the medullary surface (Figure 4k) and in the medullary raphe (Figure 4l) compared to epilepsy and non-epilepsy control groups (Patodia et al., 2019). Although functional studies are not feasible in post-mortem tissue samples, their morphology, distribution and immunophenotype are in keeping with specialised glia with respiratory modulatory roles (SheikhBahaei et al., 2018a). Alteration in their distribution and density could also be of relevance to the concerted re-organisation of medullary respiratory networks in epilepsy.

5. High-risk genes for SUDEP and neuropathology findings

Recent genetic studies have demonstrated heterogeneity, complexity and potentially oligogenicity (interactive influence of a small number of genes) in SUDEP (Goldman et al., 2016). SUDEP risk genes identified so far mainly align with known epilepsy genes e.g. *SCNI1A,* associated with Dravet syndrome (Shmuely et al., 2016) and *DEPDC5* associated epilepsy (Ribierre et al., 2018), or cardiac-epilepsy genes, for example epilepsy associated with long-QT syndrome, which arguably represent ‘cardiac-SUDEP’; there is less evidence for SUDEP genes independently influencing respiratory or autonomic systems (Friedman et al., 2018). Future research exploring if SUDEP genetic risk factors predict distinct mechanisms of death, and therefore personalized prevention strategies, is essential. For example, in a *depdc5* mouse model, SUDEP was prevented through inhibition of MTORC1 (Klofas et al., 2020).

An important goal of future neuropathology, neuroimaging and clinical research is to stratify SUDEP cases according to genetic risk factors. Dravet syndrome with *SCN1A* mutations is associated with the highest rate of SUDEP (Cooper et al., 2016). In the above discussed studies, a cohort of seven Dravet cases was compared to all other non-genetically characterized SUDEP cases (Patodia et al., 2019; Patodia et al., 2018; Patodia et al., 2020b; Patodia et al., 2020c); the only difference noted was a higher TH neuronal density in the medullary raphe in Dravet cases (Patodia et al., 2020c). Clearly, larger genetically characterized SUDEP cohorts are required to dissect any differences in brainstem neuronal modulation.

6. Central autonomic networks connecting to the brainstem

The central autonomic network (CAN) refers to the supratentorial brain regions involved in autonomic modulation with connection to brainstem centres (Beissner et al., 2013) (Valenza et al., 2020; Valenza et al., 2019) (Figure 1). These regions orchestrate physiological and volitional modulation of cardio-respiratory output e.g. during exercise, speech, emotional responses and singing (Urfy et al., 2014). This complex network and their reciprocal inter-connectivity with the brainstem, as well as regionally specific anatomical functions, are still a focus of clinical investigation. For example, recent electrical stimulation studies of the human insular cortex subregions showed direct effects on cardiac function (Sanchez-Larsen et al., 2021). The hypothesis in SUDEP is that abnormal activity in CAN regions, either during a seizure or spreading depolarisation following a seizure, is propagated or descends to the brainstem, as has been shown experimentally (Loonen et al., 2019). Functional MRI studies in SUDEP, or epilepsy patients considered at higher risk, reveal altered connectivity between autonomic cortical and subcortical cardiac and respiratory regulatory regions, suggesting reorganisation of these networks (Allen et al., 2017; Allen et al., 2019a; La et al., 2019; Tang et al., 2014). Structural MRI studies have also revealed distinct regional patterns of increased or decreased MRI grey matter volumes in SUDEP, some of which coincide with autonomic brain regions such as the amygdala and thalamus (Allen et al., 2019a; Allen et al., 2020; Ogren et al., 2018; Wandschneider et al., 2015).

6. 1 Studies of the amygdala in SUDEP

The amygdala has been a major region of research in SUDEP. The amygdala is a nuclear complex with direct and indirect connections with the brainstem respiratory nuclei, including the pre-BötC (Bzdok et al., 2013; Swanson et al., 1998; Yang et al., 2020). The lateral nucleus of the amygdala can generate spontaneous inter-ictal activity (Graebenitz et al., 2011) and seizure propagation to the amygdala is associated with apnoea in some (Nobis et al., 2019) but not all case studies (Park et al., 2020). Electrode stimulation of the amygdala region in patients undergoing investigations for epilepsy (Dlouhy et al., 2015; Lacuey et al., 2017; Nobis et al., 2018) resulted in apnoeic episodes. These occurred following stimulation of the medial amygdala (Nobis et al., 2018) and lateral and basolateral nuclei (Dlouhy et al., 2015). More recently, a study in paediatric epilepsy patients has localised a specific amygdala region, overlapping the basolateral, basomedial, cortical medical nuclei and intercelated nuclei, associated with inhibiting respiratory activity (Rhone et al., 2020).

An earlier post-mortem study investigated pathology of the central nucleus in SUDEP, in view of the greater evidence for its direct connections with respiratory brainstem nuclei (Yang et al., 2020). Lower neuronal densities and increased astrocytic densities were noted in lateral nucleus of amygdala but not the central nucleus compared to normal controls (Thom et al., 1999). However as there was no epilepsy control group the specificity of this sclerosis pattern for SUDEP was uncertain. In a further immunohistochemistry study, Michalak et al., reported no significant difference between SUDEP, epilepsy controls and normal controls in amygdala astroglial populations as well as microglial densities (using CD163 and HLA-DR immunolabelling) and also no evidence of blood brain barrier

breakdown as markers of acute neuropathology in SUDEP (Michalak et al., 2017). The amygdala is enriched in galanin (Gentleman et al., 1989; Perez et al., 2001), NPY (Adrian et al., 1983) and SST (Geola et al., 1981) compared to other brain regions. A quantitative immunohistochemistry study showed a reduction in galanin in the lateral nucleus in SUDEP cohorts compared to normal controls (Somani et al., 2020). Interestingly, all neuropeptides studied (galanin, NPY and somatostatin) were higher in epilepsy-controls than both SUDEP cases and normal controls indicating depletion in SUDEP compared to relative augmentation of networks in chronic epilepsy (Somani et al., 2020). One interpretation is that neuropeptide consumption or "exhaustion" occurs in SUDEP, resulting in neuronal fatigue with consequent effects on amydgala-brainstem networks.

More recently the mesial temporal lobe structures and amygdala have been investigated in SUDEP for adenosine receptors and its clearance mechanisms (Patodia et al., 2020a). Adenosine is a suppressor of seizure activity and candidate molecule in SUDEP (Tescarollo et al., 2020). The adenosine hypothesis of SUDEP proposes that fatal respiratory depression and impaired arousal are mediated through the adenosine receptors in autonomic and brainstem centres in the post-ictal period (Boison, 2012; Erlichman et al., 2010; Scislo et al., 2005; Shen et al., 2010; Zhang et al., 2013). Astrocytes regulate adenosine levels during and after seizures with astroglial adenosine kinase (ADK), the major metabolic clearance route (Weltha et al., 2018). Surgical tissues from patients who had undergone resective surgery for temporal lobe epilepsy with hippocampus sclerosis were used, and the cases risk stratified for SUDEP according to the frequency of generalised seizures pre-operatively. Quantitative immunohistochemistry for ADK in the amygdala revealed similar findings across SUDEP risk groups. However, significantly increased adenosine receptor A_1R in the amygdala but lower $A_{2A}R$ in the epilepsy patients regarded as high risk for SUDEP compared to low risk was seen (Patodia et al., 2020a). Of relevance, tolerance to hypoxia is mediated through A_1R (Fredholm et al., 2011) and patients with apnoea associated with amygdala stimulation characteristically show no dyspnoeal symptoms (Dlouhy et al., 2015; Rhone et al., 2020). Furthermore, adenosine is implicated in the prolonged depression of synaptic transmission after spreading depolarization via A_1R receptor activation (Lindquist et al., 2012). Spreading depolarisation to the brainstem has been shown to mediate irreversible respiratory collapse in experimental SUDEP genetic models (Aiba et al., 2015; Loonen et al., 2019) although not in wild-type animals (Jefferys et al., 2019). In some experimental SUDEP models depolarisation spreads through the amygdala prior to brainstem progression (Loonen et al., 2019); observed alterations in adenosine receptor levels and distribution in both the amygdala and brainstem may therefore be of relevance.

The amygdala is also highly enriched in serotonin networks from the midbrain raphe (Beliveau et al., 2017) and SERT immunohistochemistry has been used as specific tissue marker to demonstrated and quantify serotonergic afferent networks (Asan et al., 2013). Serotonergic axons have been shown to both regenerate following injury in addition to compensatory sprouting from non-injured axons therefore demonstrating a unique potential for repair and remodelling (Kajstura et al., 2018). In a post-mortem series, increased SERT immunolabelling in the basal and accessory basal nuclei of the amygdala and peri-amygdala cortex in SUDEP compared to epilepsy controls as well as higher hippocampal SERT in TLE patients at higher risk for SUDEP has been observed (Patodia et al., Submitted).

Localised increased SERT in the amygdala in SUDEP may have again have functional implications through reduced availability of 5-HT in the vulnerable post-ictal period, deregulating critical intra and inter-amygdala connections.

6. 2 The hippocampus in SUDEP

The hippocampus is recognised to have autonomic regulatory functions and connections with the brainstem (Arrigo et al., 2017; Edlow et al., 2016) and experimental stimulation confirms hippocampal modulation of respiratory and cardiovascular activity (Ajayi et al., 2018) (Lacuey et al., 2017). Sudden unexplained deaths in infancy and childhood, share some circumstantial similarities with SUDEP, and developmental anomalies of the hippocampus have been reported by several groups, including malrotational and granule cell layer broadening as a potential disease biomarker (Kinney et al., 2009; Kinney et al., 2015; Kinney et al., 2016; Kon et al., 2020). This was a basis for a similar study in SUDEP, particularly as similar hippocampal cytopathological alterations occur in temporal lobe epilepsy (Blumcke et al., 2013). In a series of 187 adult post-mortems, morphometric evaluation of the hippocampus shape and size showed that, although malrotational abnormalities of the hippocampus were more frequent in SUDEP, these were not significantly increased compared to control groups (Somani et al., 2019). In addition, the granule cell layer thickness (excluding cases with hippocampal sclerosis) was increased in the epilepsy control group but not in SUDEP, indicating a lack of an association of this developmental or ‘neo-migratory’ abnormality with SUDEP (Somani et al., 2019). A significantly increased parahippocampal gyrus length was however noted in SUDEP (Somani et al., 2019), in keeping with MRI observations showing increased grey matter volumes of this region (Wandschneider et al., 2015) (Allen et al., 2019b).

6. 3 Neocortical and subcortical regions in SUDEP

Although dysfunction of higher central autonomic networks has been proposed in SUDEP from structural and functional imaging studies (Allen et al., 2019a) there is, yet limited understanding of the underlying cellular pathology. Recently investigations of Iba1-expressing microglia as a marker of seizure activity in fourteen cortical and subcortical brain regions from post-mortem cases noted significantly regionally increased microglia, including the parahippocampal gyrus, superior temporal gyrus and pulvinar in SUDEP (Somani et al., 2021). These brain regions have recognised roles in cardio-respiratory regulation (Arrigo et al., 2017; Edlow et al., 2016) (Beissner et al., 2013; Harper et al., 2005; Koos et al., 2004; Macey et al., 2005) (Yang et al., 2018). Therefore the identification of inflammatory pathways activation is of potential relevance to both regional cortical dysfunction as well understanding the cellular basis of atrophy observed on MRI (Wandschneider et al., 2015) and warranting further investigation.

7. Future directions and conclusions

The limitations and challenges of post-mortem studies in epilepsy and SUDEP, as well as the importance of investing in future systematic state of the art biobanking to optimise sample collection and stratification for future research studies in epilepsy, has been previously reviewed (Thom et al., 2017). Epilepsy of many causes can result in SUDEP but whether

there are common or distinct pathways of autonomic dysfunction leading to SUDEP, is as yet unproven. Quantitative regional immunohistochemistry studies, as described above, are an exploratory starting point that need to be further corroborated in further study designs to identify candidate cellular mechanisms. Single cell approaches, eg for somatic mutations, in addition to germline mutations (eg for *DEPDC5* epilepsies (Ribierre et al., 2018)) in critical autonomic regions as the brainstem may be insightful. Fuller genetic characterization of study groups is essential to correlate with neuropathological findings. Regional 'omics' studies, including proteomics and transcriptomics, for metabolic pathways elucidation in autonomic regions are needed to validate immunohistochemistry findings. Indeed, such studies using proteomics methods are underway (Leitner et al., 2021) and will become more feasible in the future with advancing multiplexing techniques. Larger, clinically and neuropathology defined SUDEP cohorts need to be investigated, stratified according to known ictal-autonomic dysfunction during life or if a competing cause of death or neuropathology lesion is identified at post-mortem and factoring for the circumstances and findings at death, for example if found in a compromised prone position and the level of pulmonary and cerebral oedema. Comparisons with different clinical control groups could provide insight, for example cases with chronic respiratory diseases in addition to clinically well-characterised epilepsies with similar seizure severity but without SUDEP, to enable comparison of brainstem hypoxic versus seizure-related neuroplasticity. Extending similar examination to interconnected brainstem and cortical autonomic regions, as well as non-autonomic regulatory regions, is also essential to evaluate the extent and specificity of any pathological changes.

The ultimate goal is the further identification of specific disease biomarkers for SUDEP, the clinical screening of at risk patient groups, and for exposing potential treatment targets of pharmacologically modifiable and reversible pathological alterations. Future tissue-based studies, if invested in, can have a valuable impact in this endeavour.

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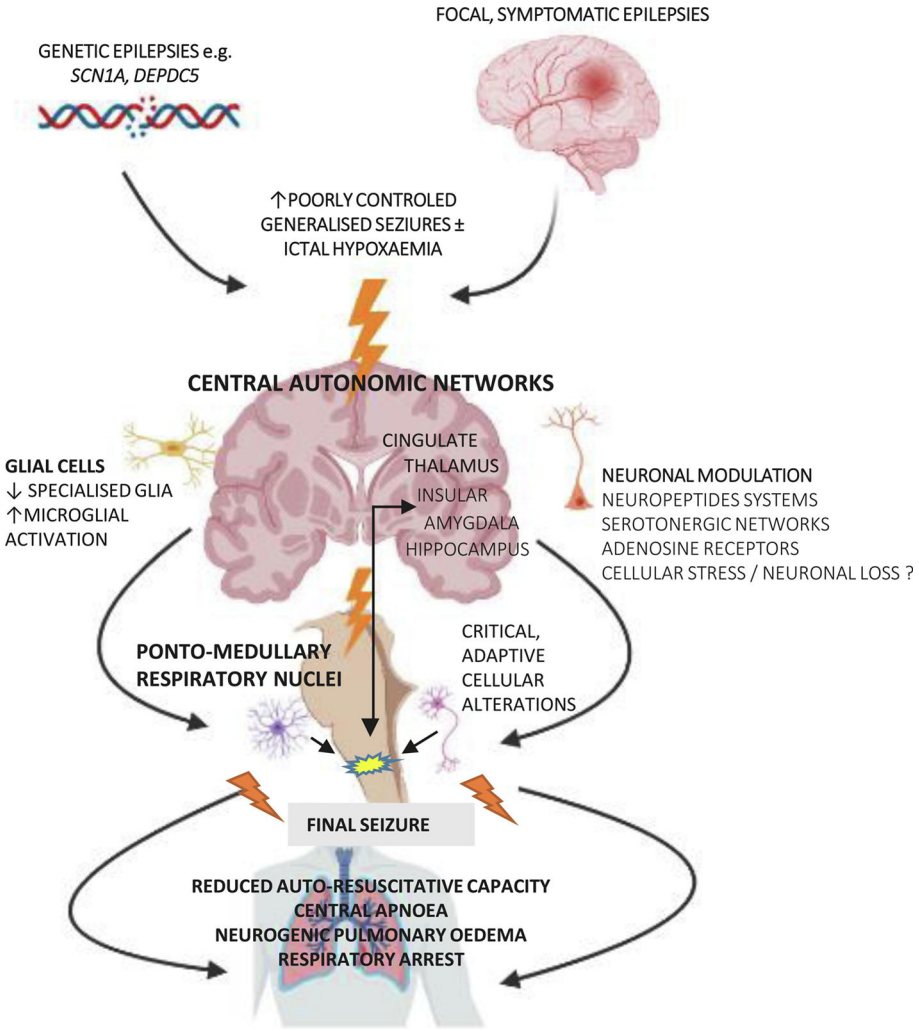

Figure 1.

Hypothesis for a common pathway of acquired seizure-related neuropathology involving autonomic regions that could culminate in increased susceptibility to SUDEP.

(The figure made using icons from *Biorender*).

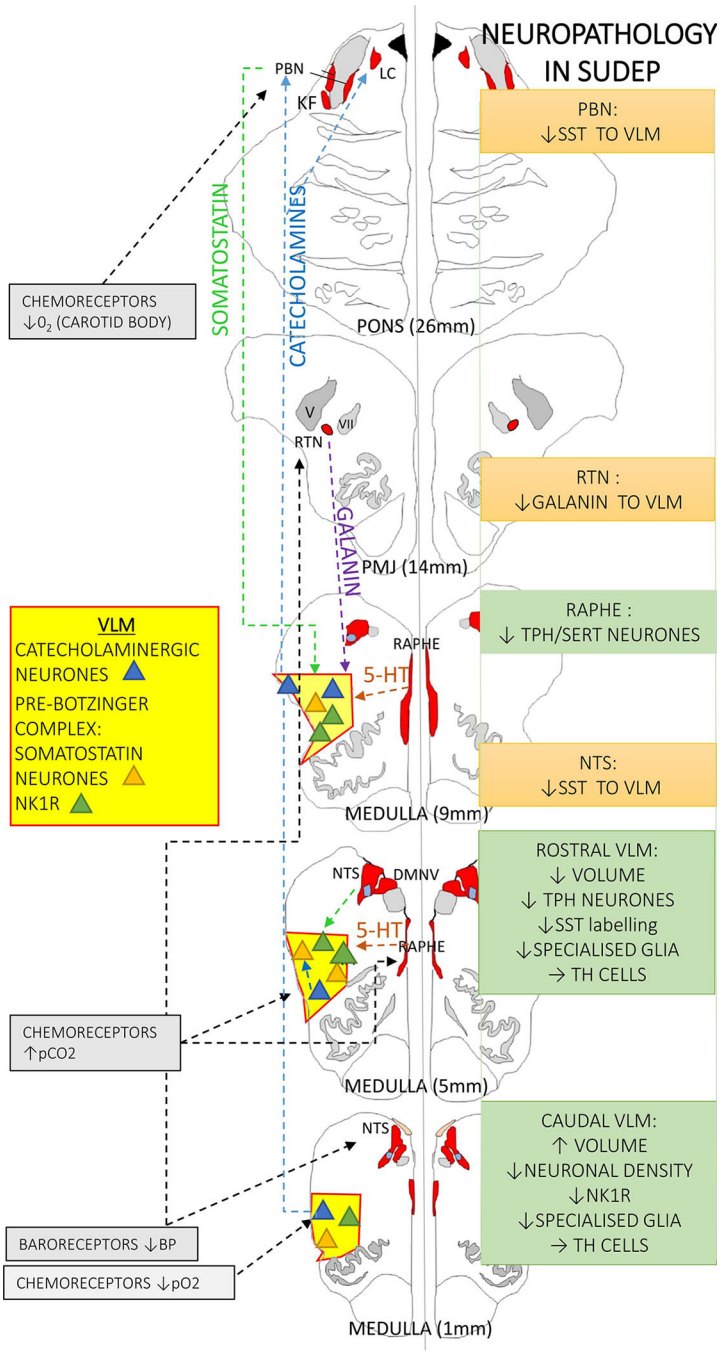

Figure 2. Medullary respiratory regulatory pathway and evidence for involvement in SUDEP.

The autonomic nuclei under study (as detailed in the text) have been highlighted only for simplicity (the autonomic and respiratory nuclei are shown in red and the ventrolateral medulla (VLM) region in yellow) and neuronal groups in VLM depicted as triangles; blue for catecholaminergic, orange for somatostatin neurones and green for NK1R positive neurones (neurokinin 1 receptor). The dashed lines indicate some of the known modulatory pathways shown on the left hand side. On the right hand side a summary of the cellular findings in SUDEP is detailed; green boxes are observations (see main text for detail)

and orange boxes are changes inferred or hypothesised from observations but requiring substantiation in further studies. TH= tyrosine hydroxylase, SST= somatostatin, PBN= parabrachial nucleus, NTS= nucleus of tracus solitarius, RTN= retrotrapezoid nucleus

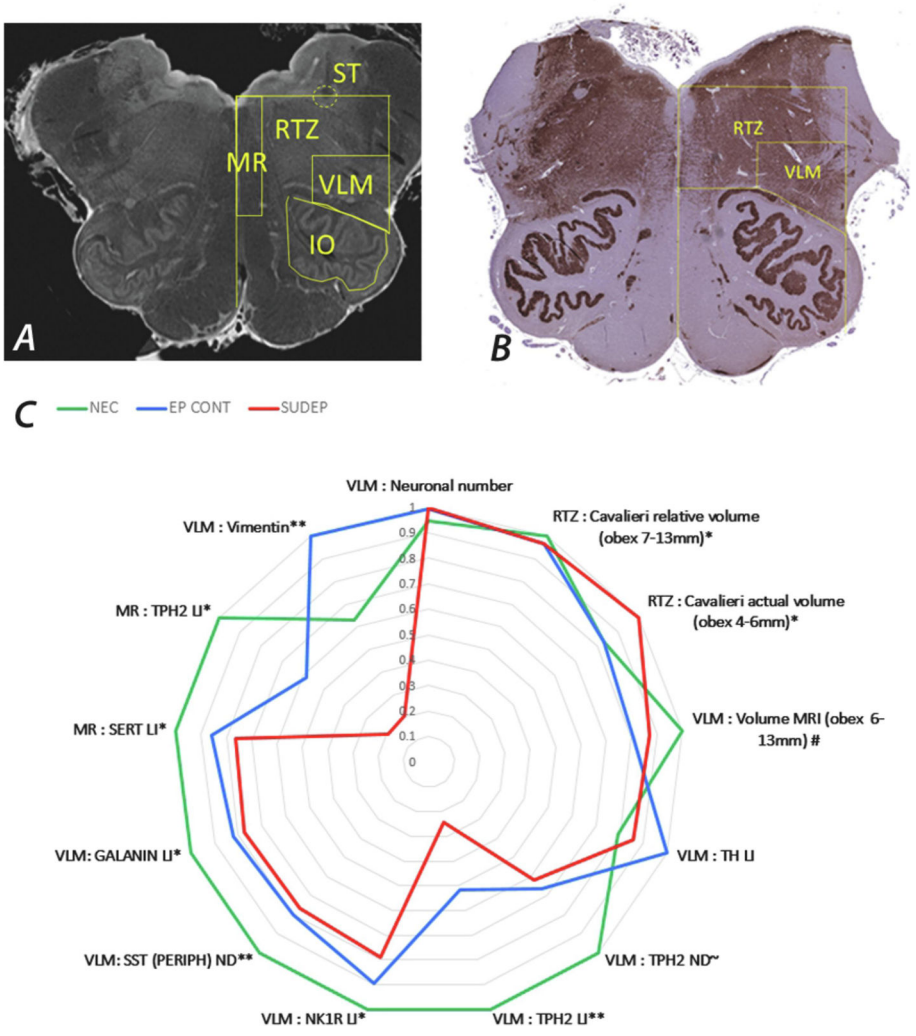

Figure 3. Summary of Brainstem quantitative studies

A. MRI of post-mortem brainstem (axial slice) using 9.4T imaging and the regions for volume estimation show; MR = medullary raphe, VLM = ventrolateral medulla, IO = inferior olive, RTZ = reticular zone and ST = solitary tract region. B. Tissue section immunolabelled with synaptophysin and showing some of the similar regions of interest outlined with anatomical co-ordinates that were used for volume estimations and cell density measurements. C. A radar plot to illustrated the mean measurements in the post-mortem group studies (red = SUDEP, blue = epilepsy (non-SUDEP) controls EP CONT and green = non- epilepsy controls). These studies are detailed in the main text. TH= tyrosine hydroxylase, TPH2 = tryptophan hydroxylase, NK1R = neurokinin 1 receptor, SST = somatostatin, SERT = serotonin transporter. ** signifies significant differences between SUDEP group and both NEC and EP CONT, *differences between SUDEP and NEC, ~ differences in SUDEP cases only noted at specific medullary obex levels (see text), # epilepsy group (blue) includes is all epilepsy cases (EP CONT + SUDEP) with significant differences shown from NEC. For the data on the MR TPH2 labelling index, the definite SUDEP cases only are shown.

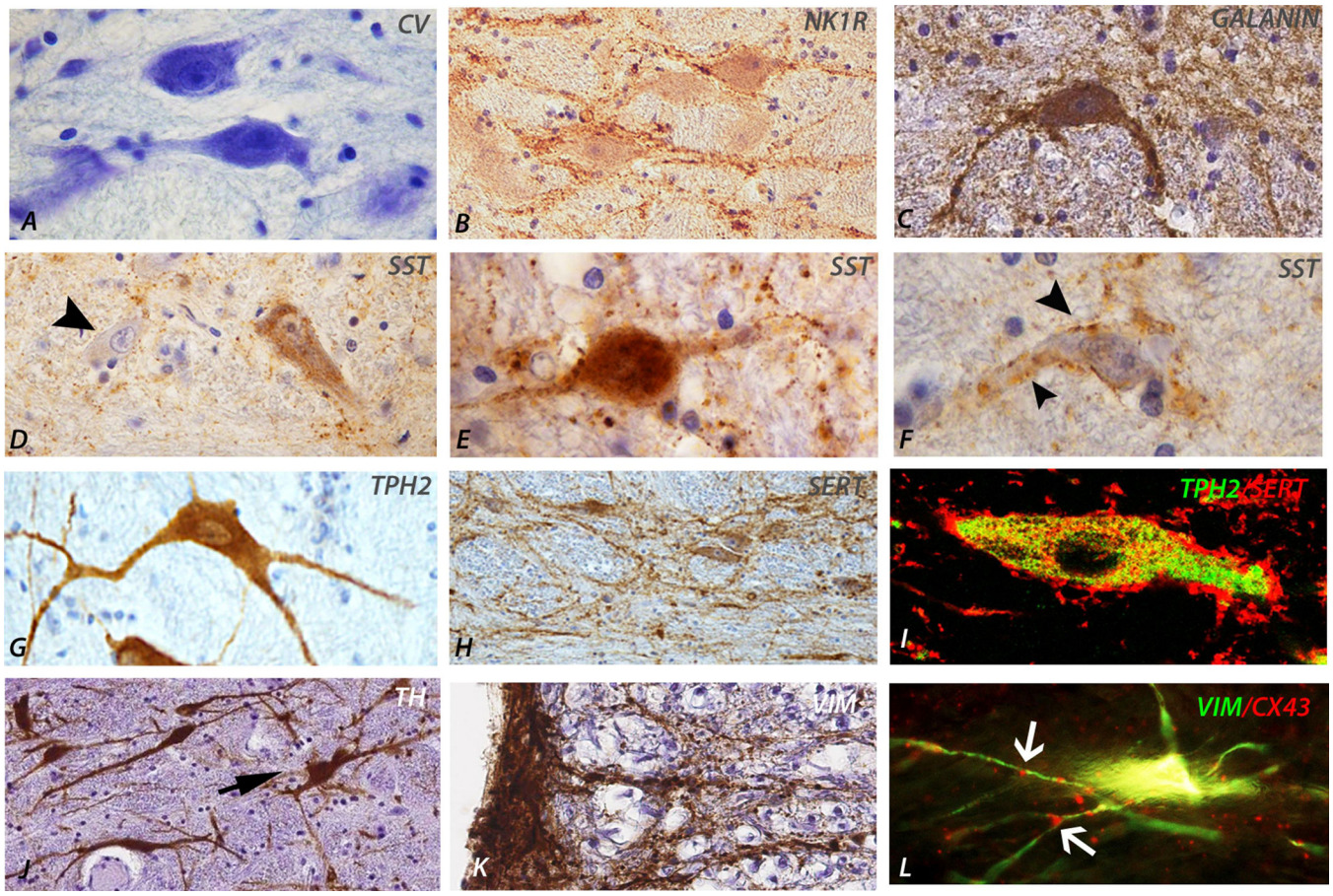

Figure 4. Medullary neuronal glial cell types and immunomarkers

A. Neurones in the ventrolateral medulla (VLM) region of medium size and stained with cresyl violet B. Neurokinin 1 receptor labelled neurones showing a peripheral rim of labelling. C. Galanin labelled neurone in the VLM region in addition to diffuse synaptic processes. D. Somatostatin (SST) labelling of neuronal populations in the VLM showing also a negatively labelled neurone (arrowed) and in E. intensely labelled neurones. F. In addition some neurones in the VLM also displayed a peripheral rim of STT labelling in keeping with synaptic input. G. Tryptophan hydroxylase (TPH2) labelling of serotonergic medullary neurones showing dense labelling of the perikarya. H. Serotonin transporter (SERT) showed a more membranous pattern of cellular labelling in the medullary raphe region. I. Double labelling of SERT and TPH2 showed cellular co-localisation in the raphe nucleus. J. Tyrosine hydroxylase (TH) labelled catecholaminergic neurones in the VLM region. K. Vimentin labelling of distinct astroglial subsets in the brainstem, including on the lateral medullary surface, extending processes in to the VLM. L. Double labelling of vimentin with connexin 43 showed distinct glial cell populations in the medullary raphe and VLM region with punctate hemichannel aggregates (arrows).