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Interleukin-6 and the serotonergic system of the medulla oblongata in the sudden infant death syndrome

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Abstract Mild infection may trigger sudden death in the vulnerable infant by cytokine interactions with a compromised medullary serotonergic (5-HT) system, leading to disrupted cardiorespiratory regulation and sleep-related sudden death. The cytokine interleukin (IL)-6 is elevated in the cerebrospinal fluid in SIDS. We tested the hypothesis that the expression of IL-6 receptors (IL-6R) and/or gp130 (involved in IL-6R signaling) is altered in the medullary 5-HT system in SIDS. Immunohistochemistry of IL-6R and gp130 was performed on medullae from 25 SIDS infants, 20 infectious deaths, and 14 controls using a semi-quantitative grading system. In the SIDS cases, mean IL-6R intensity grade in the arcuate nucleus (major component of medullary 5-HT system) was significantly higher than in the control group (2.00 ± 0.07 vs. 1.77 ± 0.08, $P = 0.04$), with no other differences in IL-6R or gp130 expression at any other site. Arcuate 5-HT neurons expressed IL-6R, indicating a site of IL-6/5-HT interaction. In SIDS, IL-6R expression is abnormal in the arcuate nucleus, the putative human homolog of rodent ventral medullary chemosensitivity sites involving 5-HT. Aberrant interactions between IL-6 and the arcuate nucleus may contribute to impaired responses to hypercapnia generated by infection (hypermetabolism) combined with rebreathing.

Keywords Cytokines · Interleukin-6 · gp130 · Arcuate nucleus of medulla · Chemosensitivity · Infection · Rebreathing · Sickness behavior

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Introduction

The sudden infant death syndrome (SIDS) is the sudden death in infancy unexplained after review of the clinical history, examination of the circumstances of death and postmortem examination [41], in the San Diego definition limited to the first year of life [23] in contrast to the original SIDS definition which uses "infant or small child" [3]. In spite of the substantial reductions in the SIDS rates with national safe (supine) sleep campaigns, SIDS remains a major public health problem and is the leading single cause of postneonatal mortality in industrialized countries world-wide [27]. It is increasingly clear that in order to eliminate all SIDS deaths, the determination of its biologic cause(s) is essential as the first step toward developing targeted preventive strategies. The triple risk model for SIDS conceptualizes sudden death as the result of three factors impinging upon the infant simultaneously, i.e., the vulnerable infant, the critical developmental period, and an exogenous stressor [8, 40]. The underlying (inherent) vulnerability may be due to a primary brainstem disorder that

makes the infant susceptible to a homeostatic stress or trigger during sleep [8, 19, 22, 32, 35]. Mild infection, especially in combination with prone sleeping position, may be important exogenous triggers [1, 36, 47]; in this regard, mild infection increases the risk 1.7-fold, prone position 10.4-fold, and the two in combination 29-fold [14]. By initiating a cytokine cascade, mild infection may trigger sudden death in an infant with an underlying pathophysiological process in the brainstem. Our work suggests that abnormalities in tissue markers of the neurotransmitter serotonin (5-HT) occur in an important subset of SIDS cases in regions of the medulla oblongata (lower brainstem); these markers concern 5-HT receptor binding, relative 5-HT transporter binding, and 5-HT neuronal number [35]. The medullary regions are involved in the modulation of breathing, blood pressure, chemosensitivity, and/or heart rate according to the level of arousal [19, 28–30, 34]. Serotonin neurons form a neural network that is located in the raphé (midline), extra-raphé (lateral), and on the ventral surface. Together with their projections to nuclei that mediate cardiorespiratory control and other homeostatic functions, e.g., hypoglossal nucleus (upper airway control) and nucleus of the solitary tract (visceral sensory input), they comprise the medullary 5-HT system [19]. The question arises; does infection in some way interact with the abnormal medullary 5-HT system in the vulnerable infant and precipitate death via cytokine interactions with dysfunctional 5-HT neurons?

We previously showed that interleukin (IL)-6 is elevated in the cerebrospinal fluid of SIDS infants [46], and that this elevation may be induced by a peripheral immune reaction [47]. Approximately one-half of the SIDS cases we have studied show signs of a mild infection, but IL-6 levels are comparable to those of infants succumbing to severe infection, suggesting an overreaction to the slight infection. In addition to its proinflammatory properties, IL-6 exerts effects outside the immune system. Non-immune cells, including neurons, can produce and secrete IL-6 [38] and express its receptor [13]. Of critical relevance to the premise that cytokines interact with central neurons to affect their function, IL-6 is shown to be important in neuronal development and the modulation of neuronal signaling [16]. In tissue sections used in this study, we have investigated both IL-6R and its signal transducer, gp130, as both are necessary for IL-6 signaling. The purpose of the following study was 4-fold: (1) to establish the baseline anatomic relationship between IL-6 and the medullary 5-HT system directly in the human infant; (2) to test the hypothesis that IL-6R/gp130 expression is altered in the neurons of the medullary 5-HT system in SIDS infants compared to infants with infectious deaths and non-infectious controls; (3) to test the hypothesis that CSF IL-6 levels are related to IL-6R and/or gp130 expression in

medullary nuclei, and that this relationship may be altered in SIDS; and (4) to determine if SIDS subgroups, particularly SIDS cases with minor infection, differentially express IL-6R and/or gp130. Furthermore, IL-6 levels in CSF were measured to verify our previous finding of elevated levels in SIDS.

To establish the baseline distribution and to explore the possibility of a potentially vulnerable period in the baseline expression of central cytokine markers, the IL-6R and gp130 expression was first studied in controls across an age span of 0–18 months postnatally. In the subsequent SIDS analysis, we focused upon medullary regions which contain 5-HT cell bodies and those that receive heavy projections. A major site of 5-HT cell bodies in the human infant brainstem is in the arcuate nucleus, the putative site for central carbon dioxide (CO_2) sensitivity in humans and animal models [10, 26, 30, 37]. In this regard, the synergistic effect of prone sleeping and infection on SIDS risk may be a "set-up" for CO_2 accumulation, as both rebreathing in the face-down (prone) position and increased metabolism due to infection may increase CO_2 levels [2, 9, 18, 51]. Death may be triggered if CO_2-sensing regions in the brainstem, such as the arcuate nucleus, are compromised and cannot mount an arousal response to protect the infant from the dangerous situation. The arcuate nucleus is also of particular interest in this study due to the previous finding by others of high neuronal IL-1β immunoreactivity at this site in SIDS cases compared to controls [17].

Materials and methods

Clinicopathological database

During the period 1984–2007, 614 sudden deaths in infants and preschool children (0–3 years) from Southeast Norway were investigated at the Institute of Forensic Medicine, University of Oslo. All the cases were diagnosed according to the Nordic Criteria for exclusion of SIDS [11], which applies the age span of "infant or small child" given in the first SIDS definition [3], and includes criteria for changes in lung, heart and brain, as well as screening for Medium Chain Acyl CoA Dehydrogenase (MCAD) deficiency and Long QT Syndrome mutations [39]. In 313 cases (51% of the database), no explanation for death was found and they were therefore classified as SIDS, 33 cases (10%) exceeding 1 year of age. There were 57 deaths due to infection, 119 deaths due to non-infectious diseases, 76 deaths due to accidents, 23 due to neglect/abuse, and 26 due to homicide. From this cohort, 25 SIDS cases (including 2 aged 395 and 526 days postnatally), 20 infectious deaths (9 septicemia, 7 pneumonia, 1 meningitis,

Table 1 Distribution of gender, age and postmortem interval in the three study groups

Study groups	n	Gender (male)	Mean age, weeks (SD)			Mean postmortem interval, h (SD)
			Postnatal	Gestational	Postconception	
SIDS	25	16 (64%)	21 (18)	39 (3)	60 (18)	25 (22)
Infectious deaths	20	13 (65%)	33 (33)	39 (2)	72 (33)	21 (15)
Controls	14	10 (71%)	30 (25)	39 (2)	69 (26)	22 (11)

There is no statistical difference among the three study groups in gender, postnatal, gestational, or postconceptional age, or postmortem interval (time between death and autopsy)

and 3 viral infections) and 14 control cases predominantly due to violent deaths were studied. The SIDS cases are described in Tables 1 and 2 and the control cases are described in Tables 1 and 3. The controls were selected on the basis of the availability of complete clinicopathological information. The infectious deaths were diagnosed on the basis of microscopic and microbiological findings (in CSF, blood and tissue specimens). Subjects with histological brain abnormalities, including hypoxic and traumatic injuries, were not included in the present study. The autopsy and death scene reports were reviewed for relevant clinicopathological information, including age, gender, history of minor infection, prone position when found, and history of prematurity. All information was not available in each case. Cerebrospinal fluid samples were obtained by suboccipital puncture at autopsy and kept at −70°C until cytokine measurements were performed.

Immunohistochemical visualization of IL-6 receptor and gp130

Tissue sections cut at 4 μm were de-paraffinized, microwave-pretreated using citrate buffer (pH 6.0), 10 min at 90°C for IL-6R and 5 min at 100°C for gp130, before being subjected to single-label immunohistochemistry using anti-IL6R (SC-661 at 1:100, Santa Cruz, Ca) and anti-gp130 (HPA010558 at 1:25, Atlas, Sweden), both rabbit polyclonal antibodies. For both antibodies, sections were blocked in PBS containing 4% goat serum for 1 h at room temperature followed by incubation in primary antibody overnight at 4°C for IL-6R and for 48 h at 4°C for gp130. Staining was detected using a DAB detection kit (Vector Laboratories, Burlingame, CA). The staining specificity was assured by pre-incubation with excess amounts of specific peptide. Immunofluorescent double-labeling to show co-localization of IL-6R and tryptophan hydroxylase (TPOH), the 5-HT biosynthetic marker, was carried out on two sections from each group using the same anti-IL-6R antibody (SC-661 at 1:50) and monoclonal anti-TPOH (PH8 at 1:1,000, Chemicon) incubated simultaneously overnight at 4°C in PBS containing 5% goat serum. For fluorescent detection, sections were incubated for 1 h at

room temperature in Alexa Fluor goat anti-rabbit 488 and Alexa Fluor goat anti-mouse 594, both at 1:1,000 (Molecular Probes, Carlsbad, CA). Immunoflorescence was visualized with an Olympus BX51 microscope (Olympus American, Inc., Melville, NY) using FTC and TRITC filters.

A semi-quantitative scoring system was developed to assess the IL-6R and gp130 immunostaining intensity in the single-labeled immunohistochemical preparations using an internal standard in each slide. The ependymal cells, known to express IL-6R [42], lining the fourth ventricle were the internal standard for IL-6R (Fig. 1) and the lateral cuneate nucleus was the internal standard for gp130 (data not shown). These standards were assigned after a complete review of all slides in a blinded fashion, demonstrating the uniformity of staining intensity of these regions across all cases and ages. Scores for the staining intensity from 0 to 3 were assigned to each nucleus where 0 = no staining, 1 = less than standard, 2 = same as standard, 3 = more than standard (Fig. 1). The staining was assessed in a blinded fashion, without knowledge of the diagnosis or age of the subject. In the dorsal motor nucleus of vagus, where a distinct difference in neuronal staining for IL-6R could be seen between the dorsal and ventral part, a separate score was given for each part. For all other nuclei, the neuronal staining was homogeneous through all subdivisions of nuclei and they were thus assigned a single score. Intensity scoring was performed in the following regions: raphé obscurus (midline site of 5-HT neurons); arcuate nucleus (ventral medulla, embedded subset of 5-HT neurons); hypoglossal nucleus (upper airway patency, receives 5-HT terminals), dorsal motor nucleus of vagus (preganglionic outflow of parasympathetic system, receives 5-HT terminals), nucleus of the solitary tract (nTS) (visceral sensory input, receives 5-HT terminals), lateral cuneate nucleus (spinocerebellar relay), nucleus ambiguous (preganglionic outflow of parasympathetic system, receives 5-HT terminals), spinal trigeminal nucleus (SpV) (pain and temperature of the head and neck, receives 5-HT terminals), and inferior olivary nucleus (cerebellar relay, receives 5-HT terminals). Sections from all three study groups were investigated

Table 2 Clinicopathological information on the subjects in the SIDS group

#	Gender	Age (weeks)		Sleeping position	Place of death	Signs of mild infection	Postmortem interval
		Postnatal	Postconception				
1	M	34	74	Head covered	Own bed, tangled in duvet	No	48
2	M	29	69	Prone	In bed with parents, feet end	No	115
3	M	21	61	Side	In bed with mom	–	8
4	F	18	58	Supine	In stroller, smoking in same room	No	10
5	M	23	63	Supine, head covered	Own bed, parents' room	No	13.5
6	F	30	67	Prone	Own bed, own room	Yes	25.5
7	F	2	42	Supine	In bed with mom, parents smoke	Yes	10
8	F	22	62	Prone	In stroller, outside	No	44
9	F	30	65	Side	Own bed, parents smoke	No	26
10	M	4	44	–	In bed with both parents	Yes	25.5
11	F	8	36	Supine	In bed with both parents	–	15
12	M	1	41	–	In bed with both parents	No	9
13	M	18	58	Prone, face down	Own bed	No	10
14	M	27	67	Prone, face down	On floor	–	34.5
15	M	17	57	Prone	On floor	Yes	8
16	M	13	49	–	In bed with mom	No	7
17	F	2	42	Prone	In bed with both parents	–	7
18	F	56	96	Supine	In stroller, inside	No	18
19	M	75	111	Prone	Own bed	Yes	9
20	M	1	41	Supine	In bed with both parents	–	29
21	M	49	82	Prone	Own bed, mom drugs/alc.	Yes	27
22	M	15	55	Supine, head covered	Own bed	Yes	27.5
23	F	3	43	Supine	In bed with both parents	No	29
24	M	9	51	Supine	In bed with mom	–	24
25	M	27	67	–	In bed with both parents	Yes	36

blindly by three observers (IJR, RH and HCK) using a three-headed microscope. Inter- and intra-observer reproducibility were tested blindly on 15 sections randomly chosen from all groups, by a fourth observer (ÅV) and re-evaluation by IJR 4 months after initial scoring. An effort was made to score the intensity of 5-HT$_{1A}$ receptor immunoreactivity in different nuclei to establish an underlying 5-HT defect in this particular database, but there was no region with consistent staining across ages and diagnoses which could be used as an internal standard. Thus, the intensity of 5-HT$_{1A}$ receptor immunostaining could not be performed.

ELISA measurement of IL-6 in CSF

Cerebrospinal fluid (CSF) from all the cases were examined with respect to IL-6, using enzyme linked immunosorbent assay (ELISA) (Biotrak Amersham), described elsewhere [46]. A small subset of the CSF IL-6 levels presented in this paper has been previously published [46, 48].

Table 3 Clinicopathological information on the subjects in the control group

| #	Gender	Age (weeks)		Cause of death
		Postnatal	Postconception	
1	F	32	72	Suffocation
2	M	48	88	Strangulation
3	F	53	93	Strangulation
4	M	0	40	Knife wound (homicide)
5	F	37	77	Strangulation
6	M	35	75	Suffocation
7	M	0	40	Cardiac arrest after bronchoscopy
8	M	24	58	Drowning
9	M	77	117	Drowning
10	F	58	96	Suffocation
11	M	0	35	Drowning
12	M	0	40	Drowning
13	M	7	47	Dehydration (neglect)
14	M	49	89	Small intestine volvulus
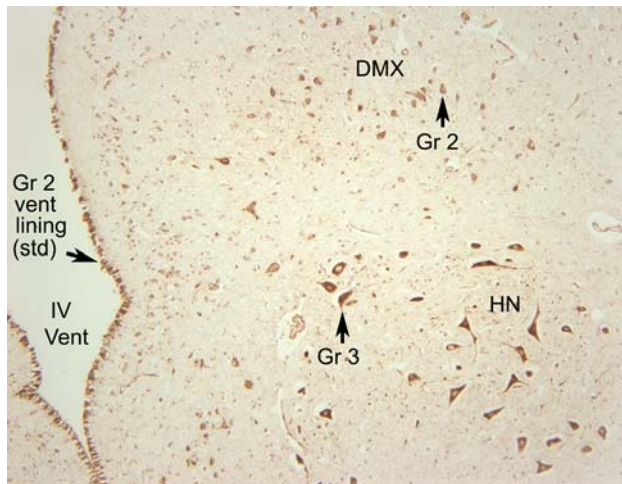
Fig. 1 A semi-quantitative scoring system from 0 to 3 was used to score the intensity of immunostaining with antibodies to IL-6R. Staining is grade 2 in the internal standard of the ependymal lining of the fourth ventricle. Relative to this standard, staining in the hypoglossal nucleus is greater (grade 3) and in the dorsal motor nucleus of the vagus, the same (grade 2). *DMX* Dorsal motor nucleus of the vagus, *HN* hypoglossal nucleus, *std* standard, *vent* ventricle

Statistical analysis

Differences in gender, age and postmortem interval among the groups, were tested using chi square for dichotomous measures and analysis of variance (ANOVA) for continuous variables. For the baseline study, non-parametric tests such as the Wilcoxon Rank Sum and Kruskal–Wallis test were used to analyze the immunohistochemical staining intensity and the effect of postconceptional age. In the SIDS study, ANCOVAs were used to test for immunostaining differences among the study groups adjusted for postconceptional age. Inter- and intra-observer reproducibility were assessed using weighted kappa-test. To test for differences in the relationship between CSF IL-6 levels and the immunostaining grade of intensity in the medullary nuclei among the groups, we first calculated the Spearman's correlation coefficient between CSF IL-6 levels and the immunostaining grade, then we used the test of equality of observed correlations in which a Z statistic was calculated to determine whether the correlations were significantly different between groups. The differences in correlations were determined between: (1) SIDS and controls; (2) SIDS and infectious cases; and (3) infectious and control cases. The Wilcoxon Rank Sum Test was used to test for significant differences between IL-6R and gp130 expression between subgroups of SIDS cases; exact P values were calculated due to the small sample sizes. The SIDS with minor infection cases had a history of "colds" or fever around the time of death. Premature infants were born less than 37 gestational weeks. For all analyses, a P value less than 0.05 was considered significant.

Results

### Baseline developmental study of IL-6R and gp130 expression

We first determined the baseline distribution of IL-6R and gp130 expression in selected medullary nuclei using only the control cases, followed by a comparative analysis of staining intensity among all three study groups (see below). In the baseline study, the staining intensity was ubiquitous and present in all neuronal subtypes in all nuclei (Fig. 2), but of variable intensity. Immunostaining for IL-6R and gp130 was noted in both the soma and processes of neurons. In certain regions, IL-6R staining intensity was on average comparable to the internal standard (grade 2) in the hypoglossal nucleus, dorsal motor nucleus of the vagus (dorsal subdivision), inferior olive, and arcuate nucleus. In contrast, it was lower on average than the internal standard in the dorsal motor nucleus of the vagus (ventral part), raphé obscurus, SpV, and nTS (Figs. 2, 3). For gp130 staining, intensity in arcuate nucleus and nucleus ambiguus was essentially the same as the standard; the dorsal motor nucleus of the vagus, hypoglossal nucleus, inferior olive, and raphé obscurus was lower, and nTS and SpV only showed weak staining (Fig. 2). This staining pattern does not change significantly throughout the first 18 months after birth. With the use of double-label immunohistochemistry in six representative controls, we found that IL-6R was localized to the soma and processes of all 5-HT neurons in the raphé obscurus and arcuate nucleus (Figs. 4, 5). In these same regions, non-5HT neurons likewise expressed IL-6R (Figs. 4, 5).

The study group characteristics and IHC expression of IL-6R and GP130

In the SIDS study, there were no statistical differences in gender, age, or postmortem interval among the study groups (Table 1). There was no adverse effect of postmortem interval upon immunostaining for antibodies used. The mean postnatal age in the SIDS group was 21 weeks with a standard deviation of 18 weeks; the mean gestational age was term (Table 1). The male gender predominated in the SIDS group (64%), as in the other study groups (Table 1). Forty-four percent of the SIDS group had a history of minor infection around the time of death; 45% were found prone and 10% side, both not recommended sleeping positions; 48% were co-sleeping (Table 2).

The mean IL-6R intensity grade in the arcuate nucleus was significantly higher in the SIDS group than in the control group (2.00 ± 0.07 vs. 1.77 ± 0.08, $P = 0.04$) (Figs. 6, 7). A trend of elevated intensity was seen in the infectious group compared to the controls, but the difference was not statistically significant ($P = 0.13$). No other significant

Fig. 2 Montage from control case of IL-6R next to gp130 in five of the regions assessed: two serotonergic regions marked in *red* and three important projection sites involved in cardiorespiratory control marked in *blue*. The two proteins are ubiquitous in distribution. Staining intensity is greater in some regions relative to others, e.g., immunostaining for IL-6R in the arcuate nucleus (low) versus the dorsal motor nucleus of the vagus (high). There are also differences in the staining intensity across nuclei between IL6R and gp130 staining, e.g., in the dorsal motor nucleus of the vagus
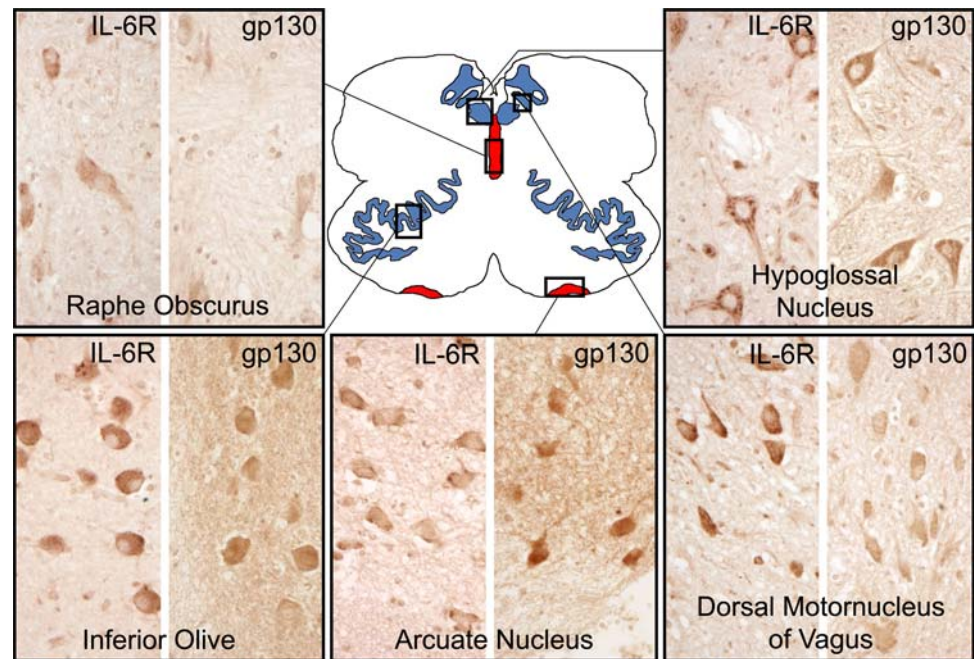
differences in IL-6R immunostaining intensity were found between the SIDS group versus the control group and infection group in any nucleus sampled. No significant difference in gp130 immunostaining was found between SIDS and controls in any of the nuclei investigated. The mean gp130 score in the arcuate nucleus, however, was significantly higher in the infection group compared to both SIDS (2.10 ± 0.14 vs. 1.68 ± 0.13, $P = 0.03$) and controls (2.12 ± 0.15 vs. 1.39 ± 0.18, $P = 0.004$) (Fig. 7). The infection group also showed higher staining intensity for gp130 in the SpV compared to SIDS (0.42 ± 0.10 vs. 0.10 ± 0.09, $P = 0.03$). Weighted kappas ranged from 0.72 to 0.77 indicating good agreement between observers.

CSF levels of IL-6

There was a marginal difference in IL-6 levels in the CSF between the SIDS group (median 14.00 pg/ml, range 0–5471) and the control group (median 7.00 pg/ml, range 0–689, $P = 0.08$) (Fig. 8). In contrast, there was a significant increase in the levels in the infectious group (median 46 pg/ml, range 0–912) compared to the control group ($P = 0.04$) (Fig. 8). The SIDS values were intermediate between those of the infectious and control groups.

Relationships between CSF IL-6 levels and IL-6R or gp130 expression

The main impression of the correlation studies of CSF IL-6 levels and brainstem expression of IL-6R and gp130 was the observation of trends for negative relationships in the
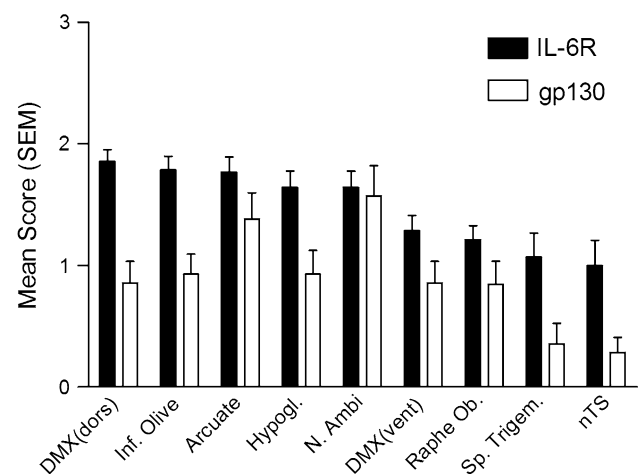

Fig. 3 Graphical depiction of the relative baseline distribution (control cases only) of IL-6R (*black*) and gp130 (*white*) expression in components of the medullary 5-HT system in the control cases. The relative distribution is dissimilar between the two proteins. *DMX (dors)*, dorsal subdivision of the dorsal motor nucleus of the vagus, *inf. olive* inferior olive, *hypogl.* hypoglossal nucleus, *N ambi* nucleus ambiguous, *DMX (vent)* ventral subdivision of the dorsal motor nucleus of the vagus, *Raphe Ob* raphé obscurus, *Sp. Trigem.* spinal trigeminal nucleus, *nTS* nucleus of the solitary tract

infectious deaths and in the controls, whereas the opposite was seen in the SIDS group. The results of the correlation studies are therefore presented as correlation differences (Tables 4, 5). We found that there were significant differences in the correlations between the SIDS and control group (Table 4) and between the SIDS and infection group (Table 5) in the majority of nuclei sampled in the medulla for both IL-6R and gp130. In infectious and control groups, there was a negative

Fig. 4 Serotonergic neurons, labeled by the biosynthetic enzyme for serotonin, tryptophan hydroxylase (*TPOH*) (*middle*, *stained red*) express IL-6R (*left*, *stained green*) in the raphé obscurus, the major component of the medullary 5-HT system, in control case. The merged image of these two panels (*right*) demonstrates the co-localization of TPOH and IL6R
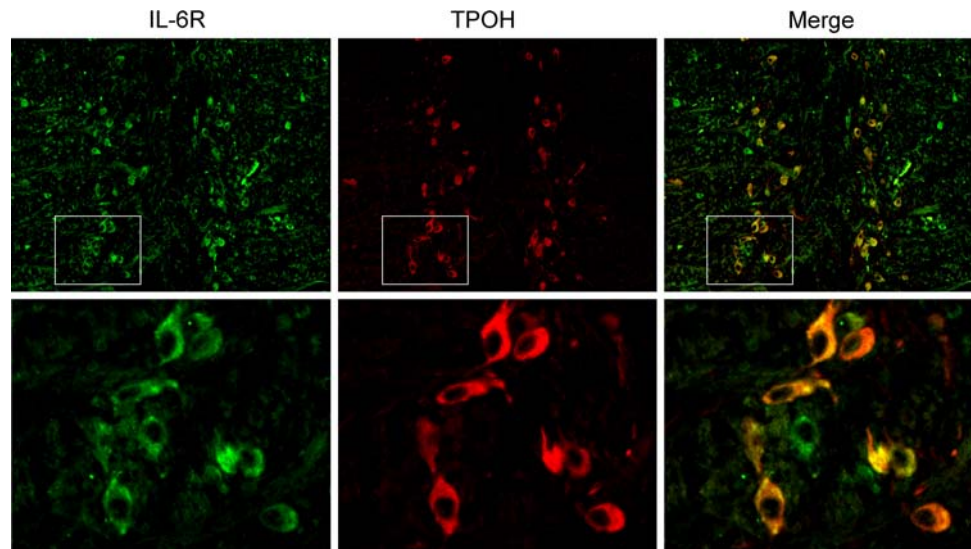

Fig. 5 A 5-HT neuron, labeled with TPOH (*middle*, *stained red*) expresses IL-6R (*left*, *stained green*) in the arcuate nucleus at the ventral medullary surface in control case. The merged image of these two panels (*right*) demonstrates the co-localization of TPOH and IL6R
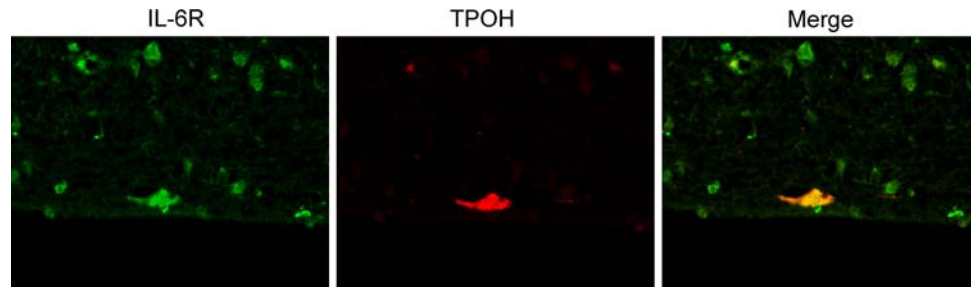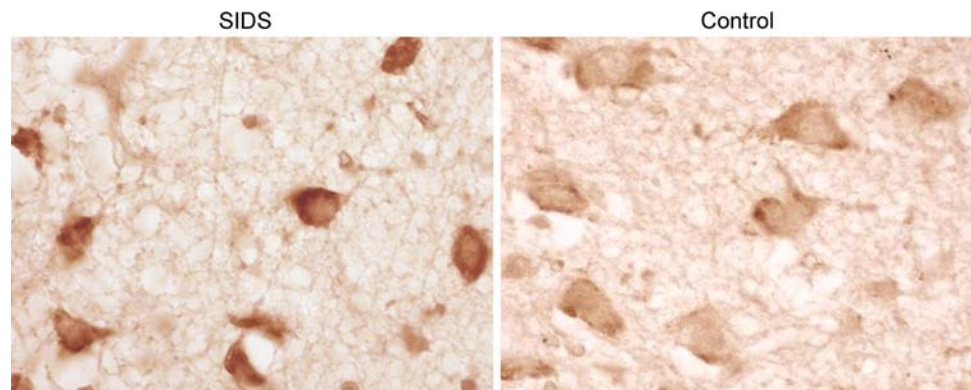

Fig. 6 In the arcuate nucleus, IL-6R staining intensity is visually higher in a representative SIDS case compared to a control. On average, the SIDS group was significantly higher than the control group (2.00 ± 0.07 vs. 1.77 ± 0.08, $P = 0.04$)

correlation between IL-6 CSF levels and immunostaining of gp130 in the arcuate nucleus; in contrast, there was a positive correlation in the SIDS cases (Table 4; Fig. 9).

Analysis of SIDS subgroups and IL-6R and gp130 expression

In the SIDS cases with minor infection before death ($n = 8$) compared to SIDS cases with no signs of infection ($n = 10$), there was no significant difference in IL-6R or gp130 expression, although there was a trend ($P = 0.05$) toward lower IL-6R expression in the SIDS cases with

minor infection in the dorsal motor nucleus of the vagus (ventral component). There was no difference in immunostaining intensity for either marker specifically in the arcuate nucleus ($P = 0.99$). There were also no significant differences in IL-6R or gp130 expression in prone ($n = 10$) versus non-prone ($n = 7$) SIDS cases, preterm ($n = 5$) versus full-term ($n = 20$) SIDS cases, or male ($n = 16$) versus female ($n = 9$) SIDS cases ($P \geq 0.05$). Group sizes in the subgroup of SIDS prone with infection ($n = 4$) versus SIDS prone with non-infection ($n = 4$) versus SIDS non-prone with non-infection ($n = 2$) were small and precluded, in our opinion, in the statistical analysis.
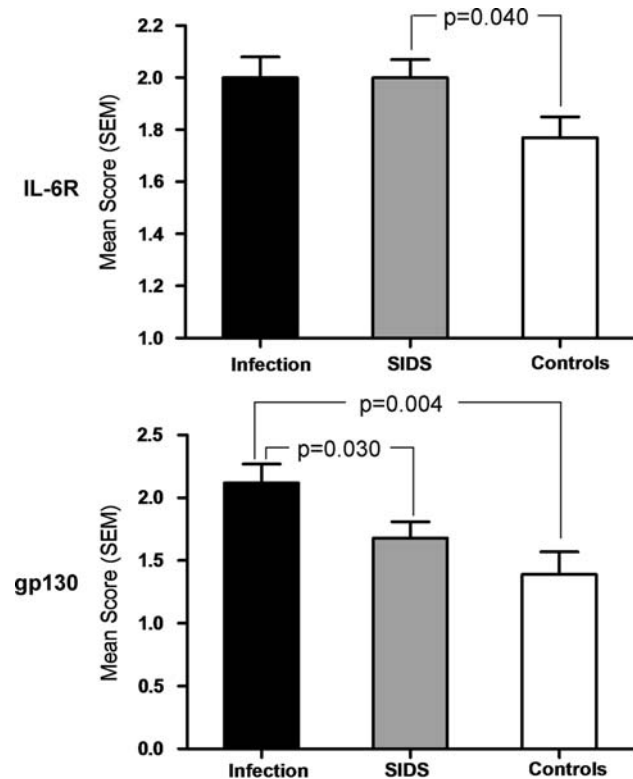
Fig. 7 The infection and SIDS groups have similar means for IL-6R. This is, however, not the case for gp130 where the infection group is significantly higher than in SIDS
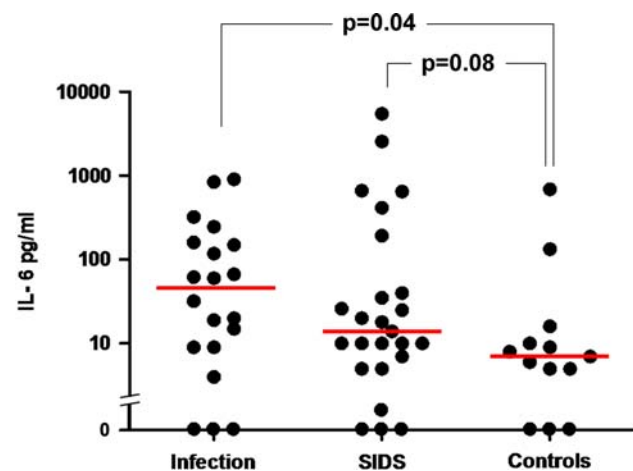

Fig. 8 Scatter plot of IL-6 CSF levels (median shown in *red*) in the three study groups replicating our previously published finding [46] that SIDS have elevated levels of IL-6, although only marginally significant compared to controls in this dataset. In one control case there was an insufficient amount of CSF to perform IL-6 analysis

Discussion

During infection, peripherally produced cytokines may cross the blood–brain barrier, bind to endogenous cytokine receptors on neuronal populations that mediate stress responses in the hypothalamus and/or brainstem, and thereby determine sickness behavior, including blunted arousal and depressed respiration [5, 12, 49]. We speculated here that a cascade of cytokines induced by a slight infection, and perhaps augmented by underlying genetic susceptibilities in cytokine metabolism [6, 31], could trigger sudden death in a vulnerable SIDS infant with an underlying abnormality in the medullary 5-HT system and/or other systems that mediate stress responses. Previous work in our group has demonstrated that at least one-half of SIDS infants have medullary 5-HT-related abnormalities [19, 22, 32, 35]. These abnormalities may lead to sudden death when the affected infant is challenged by a stressor during sleep or during one of the many transitions into and out of sleep that occurs during the night or nap-time [19, 22]. Epidemiological studies suggest that infection may constitute such a stressor [36] and this possibility is further substantiated by us through demonstration of elevated IL-6 levels in CSF of about one-half of SIDS cases [46]. It is therefore of interest to study the interplay between cytokines and their receptors and the medullary 5-HT system in SIDS. In this study, we found elevated IL-6R expression in the arcuate nucleus, a key component of the medullary 5-HT system, in SIDS compared to controls. In the following discussion, our findings are highlighted in the context of their potential clinical significance. We begin with a consideration of the possible limitations of the study.

Potential limitation of the study

A major focus of this study is the grading system for immunostaining intensity for IL-6R and gp130 expression. The quantitation of immunostaining is difficult and fraught with concerns [45]; various methods, none of which are totally satisfactory, have been put forward, including the use of computer-based imaging to assess intensity. In this study, we sought a simple and straightforward technique with an internal standard for comparison among study groups. We found after blinded review of all tissue sections that the standards we chose for each antibody stained consistently across age, diagnosis, and CSF IL-6 levels (the latter data not shown). For this reason, we felt justified in the use of the standards which were able to discern visually obvious and/or statistically significant grades between the SIDS, infection, and controls groups. Both inter- and intra-observer reproducibility were good [24], reinforcing the validity of the selected scoring method.

Baseline expression of IL-6R in the medullary 5-HT system

In SIDS research, the determination of normal development is mandatory for the establishment of pathology [8,

Table 4 Test of equality of observed correlations between CSF IL-6 levels and immunostaining intensity of gp130 or IL-6R: SIDS versus controls

Region	gp130			IL-6R		
	SIDS $n = 24$	Control $n = 14$	P value	SIDS $n = 25$	Control $n = 14$	P value
	r_s	r_s		r_s	r_s	
Arcuate nucleus	0.24	−0.23	0.01	ND	−0.39	ND
Raphé Obscurus	0.06	−0.30	0.03	0.16	−0.74	<0.0001
nTS	0.01	−0.64	<0.0001	−0.09	−0.45	0.01
DMX	0.31	−0.36	<0.0001	0.46	−0.49	<0.0001
NA	0.19	−0.19	0.01	0.56	0.20	0.01
Hypoglossal nucleus	0.25	0.23	0.89	0.21	−0.23	0.005
Inferior olive	0.54	0.10	0.003	0.19	−0.02	0.15
SpV	0.02	−0.39	0.005	−0.09	−0.27	0.22

ND Analysis not done due to entire group having the same value, *nTS* nucleus of the solitary tract, *DMX* dorsal motor nucleus of the vagus, *NA* nucleus ambiguous, *SpV* spinal trigeminal nucleus, r_s Spearman rank correlation coefficient

Table 5 Test of equality of observed correlations between CSF IL-6 levels and immunostaining intensity of gp130 or IL-6R: SIDS versus infection group

Region	gp130			IL-6R		
	SIDS $n = 24$	Infection $n = 20$	P value	SIDS $n = 25$	Infection $n = 20$	P value
	r_s	r_s		r_s	r_s	
Raphé Obscurus	0.06	0.04	0.97	0.16	−0.22	0.001
nTS	0.01	−0.46	<0.0001	−0.09	−0.59	<0.0001
DMX	0.31	−0.61	<0.0001	0.46	−0.40	<0.0001
NA	0.19	0.52	0.003	0.56	0.08	<0.001
Hypoglossal nucleus	0.25	0.03	0.06	0.21	−0.37	<0.0001
Inferior olive	0.54	0.50	0.72	0.19	0.10	0.39
SpV	0.02	0.18	0.16	−0.09	−0.16	0.50

ND Analysis not done due to entire group having the same value, *nTS* nucleus of the solitary tract, *DMX* dorsal motor nucleus of the vagus, *NA* nucleus ambiguous, *SpV* spinal trigeminal nucleus, r_s Spearman rank correlation coefficient

40]. Thus, we first analyzed the 14 cases that died from acute, non-infectious, known causes with ages ranging from 0 to 18 postnatal months. We found that IL-6R and gp130 are expressed ubiquitously by neurons in all regions in the infant medulla, including those critical to respiratory and autonomic control, as well as those which contain 5-HT neurons and receive 5-HT projections. We found that IL-6R expression did not vary across infancy, consistent with clinical and experimental findings concerning IL-6 production [25, 43, 44, 46], as well as a report of IL-6R expression in the early developing human brain with mRNA analysis [4]. Thus, the peak of SIDS at 2–4 months, i.e., the critical period for SIDS, does not coincide with a developmental change in the production of the IL-6 receptor complex.

The constitutive expression of IL-6R by 5-HT neurons

The presence of IL-6Rs on 5-HT neurons, as demonstrated in this study, may be the key to understand the interplay between the immune cytokine and the medullary 5HT-systems. Intraperitoneal injection of IL-6 in rats increases 5-HT metabolism in the brainstem [50], making our finding particularly interesting, as it points to a specific site of interaction, i.e., IL-6 bindings to IL-6Rs on 5-HT neurons. The demonstration of IL-6R on 5-HT neurons on the arcuate nucleus is noteworthy because these neurons are the putative homolog of rodent surface 5-HT neurons involved in chemosensitivity (see below) [7, 29, 37]. IL-6 might help modulate CO_2 sensitivity in these neurons, altering their response to higher levels of CO_2 during the course of an infection. However, the wide distribution of IL-6R and gp130 in all medullary nuclei investigated, including those unrelated to chemosensitivity, suggests a broader role for IL-6 in the coordination of brainstem responses to an infectious challenge.

Infection, chemosensitivity, and abnormal IL-6R/gp130 expression in the arcuate nucleus in SIDS

The key finding in this study is the increased expression of IL-6R in the arcuate nucleus in SIDS with a marginally significant increase in infectious deaths. The arcuate nucleus contains 5-HT and glutamatergic neurons that have been shown in animals to be critical to chemosensitivity [28, 34, 37]. It is also the site for several neurotransmitter
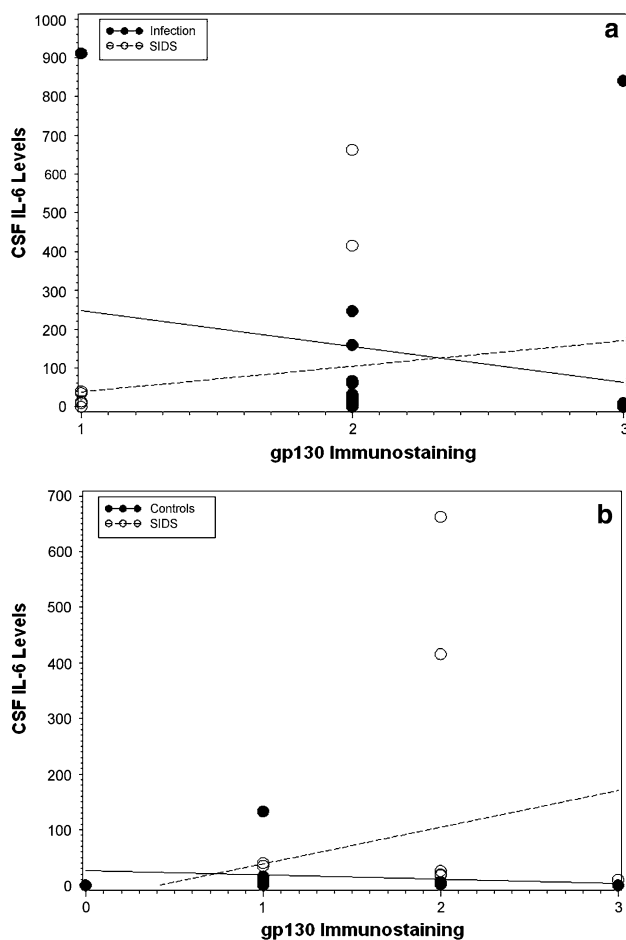
Fig. 9 These graphs depict the differences in the correlations between CSF IL-6 levels and gp130 immunostaining in two groups. The CSF IL-6 values to two SIDS cases (>1,000) are not shown in order to display the correlations clearly. **a** SIDS (*open circles*) versus infection (*solid circles*), $P = 0.008$; **b** SIDS (*open circles*) versus controls (*solid circles*), $P = 0.0002$. These graphs indicate that there are differences in the correlations between IL-6 levels in the CSF and medullary tissue gp130. These data suggest that the SIDS cases are not able to properly regulate the expression of the IL-6 receptor complex in response to infection

abnormalities in SIDS, including in 5-HT, muscarinic, and kainate receptor binding [21, 32–34]. It is well documented that CO_2 levels are elevated during severe neonatal infection [2] and, interestingly, even mild upper respiratory infection may increase CO_2 levels in infants over 3 months of age [9]. Animal studies indicate that the CO_2 elevation can be attributed to a hyper-metabolic state induced by proinflammatory cytokines [15]. In a previous study in the South-East Norway cohort, approximately one-half of SIDS cases had signs of a mild upper respiratory infection prior to death and in 20% of the cases fever was reported during the last week [1]. In this study, we found 44% of the SIDS cases had a history of mild infection, and marginally

elevated levels of IL-6 in the CSF. The theory of CO_2 accumulation caused by rebreathing in prone position [18] further substantiates the possibility of a role of hypercapnia in the lethal mechanism in SIDS. The increased expression of the IL-6R in the arcuate nucleus may be a compensatory mechanism as defective arcuate neurons may require excessive IL-6 stimulation in order to respond to altered CO_2 levels. Nevertheless, a primary deficiency in the IL-6/IL-6R/gp130-signaling pathway in SIDS infants cannot be excluded. In the infection group, there was increased expression of both IL-6R and gp130 in the arcuate nucleus, whereas there was only increased expression of IL-6R in the SIDS group, suggesting asynchrony between the receptor and the signal transducer. It is essential that gp130 be present on the cell membrane for IL-6 to elicit neuronal responses. Thus, the apparent failure to up-regulate gp130 expression in SIDS infants might render them unable to mount the adequate responses to IL-6 levels similar to those found in infectious deaths. Of note, gp130 is the signal transducer for several cytokines, thus we cannot rule out the possibility of another cytokine causing an up-regulation of gp130 in the infection group. We did not detect a difference in IL-6R or gp130 expression in the SIDS cases with minor infection compared to those without infection. We believe that this failure to detect a difference is likely due to the small sample size of the SIDS subgroups upon stratification of the presence or absence of minor infection. This issue requires further analysis in a larger dataset.

Another potential indicator of abnormal cytokine responses in the SIDS cases is the demonstration in this study of differences in correlations between IL-6R or gp130 expression and IL-6 levels. Here the analysis is based upon the premise that increasing IL-6 levels in the CSF correlate with changes in neuronal expression of the IL-6 receptor complex in the medullary parenchyma. In response to increasing CSF IL-6 levels, there may an increase or decrease in the expression of the IL-6 receptor complex, as noted in our study in the non-SIDS groups. Irrespective of the pattern of correlation in the infection and control groups; however, we found significant differences in the SIDS group. In the arcuate nucleus, for example, increasing IL-6 levels in the CSF showed a negative correlation with gp130 expression both in infectious deaths and controls. Conversely, even though gp130 expression in SIDS was low, there was a positive correlation between gp130 expression and IL-6 CSF levels. This observation may support the hypothesis of a failure in SIDS to up-regulate gp130 in the arcuate nucleus. While the cause(s) underlying this correlation, if any, is unknown, it suggests dissociations in the cellular regulation of the IL-6 receptor complex in the SIDS cases, an idea for testing in animal models.

Conclusion

The most important finding in this study is abnormal IL-6R expression in the arcuate nucleus in the SIDS cases, 44% of whom had signs of mild infection immediately prior to death. The study provides evidence for aberrant interactions in SIDS infants between IL-6 and the arcuate nucleus, a key medullary 5-HT-related region involved in protective responses to hypercapnia, potentially induced by the combined effect of prone position and mild infection. This study is in line with current research that indicates brain mechanisms underlie the pathogenesis of SIDS [20] and underscores the need for continued research in the role of the brainstem, cytokines, infection, and chemosensitivity in SIDS.

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