Research Article

Abnormal Serotonin 2B Receptor Expression in Sudden Infant Death Syndrome

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Abstract

Under the age of 12 months the Sudden Infant Death Syndrome (SIDS) is one of the central causes of infant mortality. SIDS rates in the industrialized countries vary between 0.09 in Japan and 0.8 in New Zealand per 1000 infants. However, recently, SIDS has been connected to our understanding of homeostasis, breathing rhythm, environmental and genetic risk factors as well as biochemical alterations. The serotonergic system is estimated to be involved in the etiology of SIDS as it is required for adequate respiratory rhythm generation and eupneic breathing.

In this manuscript, the 5-HT2BR expression of 34 SIDS cases and 5 controls collected at autopsy were analyzed. We found alterations in the total 5-HT2BR - positive cell count as well as the density of 5-HT2BR-positive cells in the nucleus hypoglossus in SIDS compared to controls. Moreover we found a higher 5-HT2BR- positive cell count in the lateral reticular formation in SIDS compared to controls. These alterations support the idea of 5-HT dysfunction in the brainstem which might result in breathing disturbances finally leading to SIDS.

ABBREVIATIONS


INTRODUCTION

Sudden infant death syndrome is one of the central causes of post neonatal infant mortality in children aged between one month and one year [1]. Approximately 138 boys and 90 girls are diagnosed postmortem as SIDS in Germany every year [2]. In spite of extensive research the cause of SIDS remains unknown.

More recently, research studies suggest monoaminergic systems, most notably the serotonergic system, to be involved in the etiology of SIDS [3-8]. Genetic polymorphisms in the serotonin transporter gene (5-HTT) were detected and have been linked to SIDS 5 [7]. Moreover, altered medullary 5-HT neuron count and significantly reduced 5-HT1A receptor binding density in SIDS cases compared with controls were confirmed [6]. As defined in experimental studies on rats the neurons of the serotonergic system are required for adequate respiratory rhythm generation and eupneic breathing [9-13]. 5-HT2BRs are able to increase respiratory frequency and are co expressed with 5-HT2A-receptors which modulate both respiratory frequency and amplitude [14]. A major hypothesis about the pathogenesis of SIDS is that affected infants are not able to appropriately respond to hypoxia during sleep in a critical developmental period [15]. In these infants inadequate respiratory patterns might be due to serotonergic brainstem pathology.

In the present study, we sought to evaluate whether 5-HT2BR expression might be altered in SIDS compared to controls and might support the idea of 5-HT dysfunction resulting in breathing disturbances finally leading to SIDS.

MATERIALS AND METHODS

The dataset was sourced from cases between 1998 and 2009. A total of 39 brains were analyzed. The study included three groups: 22 SIDS IA cases (median age: 4 months), 12 SIDS II cases (median age: 3 months) and 5 controls (median age: 5 months) regarding Krous’ classification of SIDS [16]. The SIDS II cases
showed, in contrast to the SIDS I cases, marked inflammatory changes not sufficient to be unequivocal causes of death.

The SIDS cases and controls were classified due to their medical records as reported by the medical examiner and in conjunction with findings in autopsy without knowledge of any histological data generated by this study. The controls included cases with (1) acute myocarditis, (2) nephritis, (3,4) heart vitium and (5) thymus hyperplasia confirmed by autopsy.

The Ethics Committee of the Georg-August-University, Göttingen, Germany approved the study and assigned the approval ID: 11/4/09 to this work. Analyses were performed blinded by the investigator to diagnosis, age and all other recorded clinicopathological variables.

Analytical procedures

Total brain and brainstem were collected at autopsy and conserved in formaline. Blocks of medullary tissue were taken from the mid-medulla at the level of the olivary nuclei corresponding to figure 20-23 according to the atlas of Paxinos and Huang [17].

Antibody generation

We used the polyclonal antibodies which were generated in our lab before and extensively described in Niebert et al. [14].

The polyclonal antibodies against the rat 5-HT2BR were generated by immunizing three New Zealand White rabbits (Charles River) with a 16mer peptide derived from the second intracellular loop of the rat 5-HT2BR amino acid sequence (NH2-CAISLDRYIAIKPIQ-COOH; NCBI-Accession No.: NP_058946). For immunization purposes, peptides were coupled to keyhole limpet hemocyanin (KLH) according to standard protocols. The rabbits were immunized with 300 µg of KLH-coupled peptide in Hunter’s adjuvant (TiterMax Gold, Sigma) five times (28-days-interval). The resulting antiserum was affinity-purified against the immunizing peptide.

Immunohistochemistry

Tissues were cryoprotected in 30% sucrose in 0.1 M phosphate buffer over night at 4°C. 40-micrometer tissue sections were collected from the blocks using a cryostat (Frigoucut, Reichert-Jung, Germany). Before any immunohistochemical treatment free-floating sections were rinsed three times in PBS (pH 7.4) for 15 minutes each. Sections were permeabilized with 0.2% Triton X-100 for 30 minutes at RT and washed twice with PBS (pH 7.4). Nonspecific binding sites were blocked with PBS containing 5% BSA for 1 hour at RT. After being washed with PBS 6 times for 5 minutes each, sections were incubated at 4°C in primary antibody solution at a dilution of 1:500 overnight and subsequently washed 6 times for 5 minutes each. Secondary antibodies (diluted 1:500) were applied for 2.5 hours at RT and rinsed with PBS 6 times for 5 minutes each. Afterwards sections were pre incubated with freshly prepared filtered diaminobenzidine (DAB) solution (500 µl: 75 mg DAB in 1, 5 ml PBS) in 30 ml PBS for 10 minutes at RT. The enzymatic reaction was started by adding 4 µl of 35% H2O2 to 3ml DAB and stopped with PBS after 5 minutes. DAB-stained sections were washed in PBS, mounted onto gelatin-coated slides, dehydrated (2 x 50% ethanol, 2 x 80% ethanol and 2 x 99.9% ethanol, 5 minutes each), cleared with four changes of xylene, cover slipped with mounting medium (DePeX from Coole Scope, Nikon).

5-HT2BR neurons were counted at standardized level of the medulla by 1 examiner using computer-based methods with Cole Scope, Nikon. Medullary levels were determined by reference to the brainstem atlas of Paxinos and Huang and corresponded to Plate 20.06b x 3mm (17). The perimeter of each section was traced and the distribution of immunoreactive cells was counted using different graphic symbols. All sections were counted twice and the mean value used for analysis. The nuclei were counted in total, not only parts of the nuclei. Statistical analysis Two-tailed, unpaired student’s t-tests were used to test for differences between SIDS and controls regarding total cell count in the nuclei examined. All statistical tests were performed at a α-level of 0.05 and carried out using Graph Pad Prism. In the diagrams significant changes are marked by asterisks (*P < 0.05). Numerical values are represented as mean ± standard error of the mean. The number of trials (n) indicates the cases investigated.

RESULTS AND DISCUSSION

Being interested in the expression pattern of 5-HT2BR in the brainstem we detected 5-HT2BR-immunopositive cells in all study cases in the external nuclear cuneatus (Figure 3), the obscural raphe nucleus (Figure 4), the hypoglossal nucleus (Figure 1) and in the lateral reticular formation (Figure 2).

The study aimed to investigate differences in between three groups: 22 SIDS IA cases, 12 SIDS II cases and 5 controls regarding Krous’ classification of SIDS [16].

There was a trend to a lower 5-HT2BR-positive cell count in the external nuclear cuneatus and the obscural raphe nucleus in SIDS IA and SIDS II compared to controls but no significant difference (Figure 5).

Comparing 5-HT2BR expression in SIDS IA to controls there was a significant alteration in total cell count and density of 5-HT2BR-immunopositive cells in the hypoglossal nucleus p = 0.02. Whereas a mean cell count of 50, 20 ± 9, 50 immunopositive cells per 0,009 cm² could be detected in the control group, only a mean cell count of 34, 91 ± 1, 94 per 0,009 cm² was observed in the SIDS IA group (Figure 5 Ab). However, the total cell count as well as the density of 5-HT2BR-positive cells is meant to be significantly lower in SIDS. These findings may suggest a hypoplasia of the hypoglossal nucleus in Sudden infant death syndrome.

The hypothesis of hypoglossal hypoplasia was confirmed analyzing all SIDS cases (SIDS IA + SIDS II) together in comparison to the control group p = 0.03. 5-HT2BR-positive cell count in hypoglossal nucleus of SIDS IA and SIDS II was about 37, 65 ± 1, 62 cells per 0,009 cm² in contrast to 50, 20 ± 9, 50 cells in the control group (Figure 5 Bb).

In contrast to all other nuclei examined there were less 5-HT2BR-positive cells detected in the lateral reticular formation in the control group compared to SIDS II p = 0.03 (Figure 5 Ch). The samples of SIDS II group showed more 5-HT2BR-positive cells in the lateral reticular formation with an average cell count.

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Figure 1: Expression of the 5HT2B-Receptor in the hypoglossal nucleus [Hyp]; horizontal section (Aa), overview (Ba), detail (Bb); Neurons of the hypoglossal nucleus (XII) revealed a strong 5-HT2BR immunoreactivity (A,B,C) that was effectively blocked after pre-incubation of the antibody with a 50-fold molar excess of the peptide CAISLDRIARKPIQ (+ peptide) that was used for immunization (Ab). Expression of the 5HT2BR in the hypoglossal nucleus in SIDS (Cb) compared to Controls (Ca); the 5-HT2BR belongs to the family of seven-transmembrane-domain receptors that are coupled to heterotrimeric guanine-nucleotide-binding protein G. The transmembrane-domains are indicated by red cylinders (I-VII). Our lab produced a monospecific polyclonal antibody against the 5-HT2BR by selecting a specific amino acid sequence (Cys146 - Gln161; NH2-CAISLDRIARKPIQ-COOH) of the second intracellular loop of the rat 5-HT2BR-sequence. The peptide exhibits 100% homology in mouse. Red letters indicate mismatches in the human-sequence (D).

Figure 2: Expression of the 5HT2B in the lateral reticular formation [Irf]; horizontal section (A), overview (Ba), detail (Bb); Expression of the 5HT2B in the lateral reticular formation in SIDS (Ca) compared to Controls (Cb).
of 48,08 ± 5.48 per 0.139 cm² compared to 26.20 ± 4.26 in the control group.

Taken together, these observations suggest 5-HT2BR expression to be involved in SIDS pathology.

Whereas the anatomical organization of 5-HT2BR has been defined in rats only [14], we demonstrate the expression of 5-HT2BR in nuclei of the human brainstem. Therefore a first value of this study was the identification of 5-HT2BR-immunopositive cells in the external nucleus cuneatus, the obscural raphe nucleus, the hypoglossal nucleus and in the lateral reticular formation. Although alterations in 5-HT1A-receptor density have been intensely studied [6], 5-HT2BR expression was never analysed in SIDS. We observed significantly less 5-HT2BR-immunopositive cells in the hypoglossal nucleus in SIDS (Ia + II) compared to controls p = 0.03. Moreover, we detected a higher amount of 5-HT2BR-immunopositive cells in the lateral reticular formation in SIDS II compared to controls p = 0.03. These results might suggest a hypoplasia of hypoglossal nucleus corresponding with findings of hypoplasia in raphe nucleus.

Figure 3 Expression of the 5HT2BR in the external nucleus cuneatus (exc); horizontal section (A), overview (B), detail (C).

Figure 4 Expression of the 5HT2BR in the nucleus raphe obscurus (rob); horizontal section (A), overview (B), details (C).
nuclei observed by Lavezzi et al. [3]. From a different point of view decreased 5-HT2BR-immunopositive cell count in SIDS might be due to a developmental deficit expressing less 5-HT2BR while 5-HT-neuron count is still normal. This hypothesis would be strengthened by a study of Paterson [6] which reveals a higher 5-HT-neuron count in SIDS compared to controls whereas 5-HT1A-receptor binding density is significantly lowered in multiple brainstem nuclei including hypoglossal nucleus. Nevertheless, molecular and cellular regulatory mechanisms between receptor expression and neuron count are incompletely understood and need to be further investigated. Animal studies support the idea that 5-HT2BR pathology could contribute to breathing disturbances occurring in SIDS. 5-HT2AR and 5-HT2BR have been shown to modulate excitatory actions on respiration [14]. Moreover, a stimulatory role of endogenous 5-HT2BR activation at the pre-Bötzing complex and hypoglossal motoneurons has been previously demonstrated [21]. Apnoe and high mortality were detected in a transgenic mouse model with a lack of serotonergic neurons [18]. Clinical studies displayed periods of apnoe, lowered cortical activity and arousal deficits in SIDS [19-21]. The correlation between clinical findings and neuropathological data support the idea that the serotonergic system is involved in the etiology of breathing disorders in SIDS and that this involvement is not only limited to the previously reported alterations in the 5-HT1AR distribution.

CONCLUSION

Current evidence suggests that SIDS is related to a convergence of stressors involved in homeostasis, breathing rhythm, environmental, genetic and biochemical risk factors. Clinical findings and neuropathological data support the idea that the serotonergic system is involved in the etiology of breathing disorders in SIDS.
disorders in SIDS. Alterations in the Serotonergic System in SIDS including less 5-HT2B receptor expression in nucleus hypoglossus in the brainstem as reported in our study might result in breathing disturbances finally leading to SIDS.

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Cite this article