Int J Legal Med (2011) 125:333–340
DOI 10.1007/s00414-010-0434-8

ORIGINAL ARTICLE

Evaluation of the agonal stress: can immunohistochemical detection of ubiquitin in the locus coeruleus be useful?

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Received: 14 October 2009 / Accepted: 19 February 2010 / Published online: 16 April 2010

Abstract The determination of the survival time after a crime as well as the concomitant physical and mental load of the victim is an important task for the forensic pathologist. The heat shock protein, ubiquitin, exerts an essential role in the cellular response to stress. We aimed to investigate the usefulness of the ubiquitin expression in the locus coeruleus as a marker for the evaluation of agonal stress. Is the amount of ubiquitin in this brain locus an indication of the length and/or intensity of the agonal period following various causes of death? The immunohistochemical (IHC) expression of ubiquitin is examined in formalin-fixed, paraffin-embedded slides of the human locus coeruleus (n=48). The evaluation of the IHC staining is blindly performed, prior to the study of the medico-legal files. According to the length of agony, a division into subgroups is made. Three possible IHC staining patterns are observed: a staining of the neuronal nucleus or the cytoplasm or both. In addition, the number of neurons with ubiquitin expression per μm^2 is calculated in each locus coeruleus. Significant differences in the number of ubiquitin-immunoreactive neurons are noticed with respect to the length of the agony: A higher density of positive neurons is seen in case of a pronounced and extended death struggle.

Keywords Agonal stress · Locus coeruleus · Immunohistochemistry · Ubiquitin · Forensic autopsy cases

Electronic supplementary material The online version of this article (doi:10.1007/s00414-010-0434-8) contains supplementary material, which is available to authorized users.

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Introduction

The process of death may continue for a certain period, during which time the agonal stress may vary. One of the important tasks for the forensic pathologist is to determine the survival time as well as the physical pressure and mental load. It is often very difficult to ascertain the duration and intensity of the agonal stress due to the poor circumstantial and pathological evidence. Even though there is no criminal law with respect to the agony, it is often an argument to influence the sentence of the judge and jury in court as well as the determination of the compensation for the next of kin. Therefore, the determination of the agonal stress is an important topic to address in current forensic practice.

Although the functional role of the locus coeruleus, which is situated in the upper pons, is still not clear, it is thought to act as an alarm system in the brain. It is also involved in activities such as reaction to stress, mood and various autonomic activities [1]. One of the central control stations of the stress system is situated in the locus coeruleus–noradrenalin system [2, 3]. Brain noradrenalin, 'the alarm system', serves on the one hand to decrease the neurovegetative functions (eating and sleeping) and on the other hand to increase the autonomic and neuroendocrine responses to stress [2, 4].

Ubiquitin, a 76-residue polypeptide heat shock protein, present in many eukaryotic cells, has multiple functions and is essential in the cellular response to stress [5–8]. Ubiquitination is essential in the cell viability and the cell cycle progression [6–9]. The essential functions of ubiquitin are to remove or repair denatured proteins produced in the cells by stress and to transport these denatured proteins to the proteolytic system [5, 7, 9–12].

Immunohistochemical (IHC) staining of ubiquitin may indicate the possible activation and redistribution of ubiquitin to form larger aggregates rather than a de novo synthesis, since there is a hypothesis that ubiquitin can move rapidly from the cytoplasm into the nucleus [13–15]. Dickson et al. researched in 1990 the ubiquitin-immunoreactive structures in the normal human brain: Immunostaining was seen both in the neurons and in the glia cells. The type of structure and the extent of staining were dependent upon the age of the individual, e.g. very few structures were ubiquitin-immunoreactive in young children [16]. Quan et al. evaluated the intra-nuclear ubiquitin-immunoreactivity in the pigmented neurons of the substantia nigra in fire fatalities [13] and in fatal acute mechanical asphyxia and drowning [14]: These authors substantiated that the stress on the central nervous system resulting from the physical activity could be evaluated by these means [13, 14]. These two articles led us to further investigate ubiquitin localisation, namely in the locus coeruleus. In addition, the midbrain periaqueductal grey matter, concerned with pain processing and modulation, undergoes overexcitation and dysfunction accompanied by the activation of the ubiquitin-system, depending on the intensity and the duration of the pain [17, 18].

In this study, we aimed to establish a relationship between the amount of neuronal ubiquitin in the locus coeruleus and the agonal stress period. The amount of ubiquitin present in the locus coeruleus is semi-quantitatively evaluated by an IHC technique. The cases studied are classified according to the estimated agony period and divided into subgroups, based on Madea [19]. The number of ubiquitin-immunoreactive neurons in the locus coeruleus is statistically analysed in order to objectify possible inter-subgroup differences.

Materials and methods

Immunohistochemistry

Brain dissections were performed after a fixation time of about 4 weeks in buffered formaldehyde (8%). Paraffin-embedded transverse tissue sections (4 μm, rotation microtome, microTec, Slee Technik CUT 4060) of the locus coeruleus from 48 forensic autopsy cases were used.

A polyclonal rabbit anti-ubiquitin antibody provided by Dako (Glostrup, Denmark, code Z0458) was used in a dilution series ranging from 1:300 to 1:600. Antigen-antibody binding was visualised by means of the catalysed signal amplification (CSA) II biotin-free tyramide Signal Amplification system (Dako, Glostrup, Denmark, code K1497). For this study, a few adjustments to the original protocol, provided by Dako [20], were performed (see Table 1).

In each protocol, appropriate negative control tissue sections were included, where the primary antibody was replaced by rabbit IgG (Dako, Glostrup, Denmark, code X0936) at a concentration dilution equivalent to that of the diluted primary antibody or by buffer (Dako 10% tris-buffered saline with Tween (TBST 20, 10× concentrate; Dako, Denmark, Glostrup, code S3306)).

Following deparaffination, rehydration and the immunohistochemistry protocol, a counterstaining with Mayer's haematoxylin (Sigma Aldrich, Bornem, Belgium) was performed.

The IHC staining of the ependymal cells [13, 14] and the corpora amylacea [21] served as an internal positive control.

Screening

With the aid of the software programme AxioVision (Rel. 4.7.1.0, copyright © 2006–2008 Carl Zeiss Imaging solutions GmbH), a digital microscope photograph was taken of a randomised part of the locus coeruleus (AxioCam ICCc3, microscope Leica DMLB). With the aid of the software programme Adobe Photoshop CS4 (version 11.0 © 1990–2008 Adobe Systems Inc.), the total number of neurons was counted in a marked out region, with known surface area. Subsequently, the number of neurons with ubiquitin-immunoreactivity (positive cells) was counted, subdivided into the number of neurons

Table 1 IHC staining procedure, adjusted after the original protocol provided by Dako [20]

Step no.	Reagent	Incubation time	Rinsing
Step 1	Peroxidase block	15 min	Buffer bath (5 min)
Step 2	Protein block	15 min	
Step 3	Primary antibody or negative control agent	Overnight in refrigerator	Buffer bath (5 times 5 min)
Step 4	CSA II rabbit link (Dako, code K1501)	15 min	Buffer bath (5 times 5 min)
Step 5	Amplification reagent	15 min	Buffer bath (5 times 5 min)
Step 6	Anti-fluorescein-HRP	15 min	Buffer bath (5 times 5 min)
Step 7	Substrate-chromogen	2 min	
Step 8	Hematoxylin counterstain	1–2 min	

containing ubiquitin reactivity solely in the nucleus, solely in the cytoplasm or neurons with ubiquitin reactivity both in the nucleus and the cytoplasm. The counting was carried out by three independent researchers by using the same microscope, after which the intra-class correlation coefficient (ICC) and the mean were calculated. The total number of neurons per μm^2 and the number of positive cells per μm^2 were calculated. The IHC evaluation was performed prior to the study of the medico-legal files.

Medico-legal files

In the 48 cases, the following variables were considered: gender, age, postmortem interval (PMI), cause, mechanism and manner of death, and possible attempt to reanimation.

The length of the agonal period was determined, based on the classification of Madea [19]. A subdivision was made by using the combined but independent opinion of three researchers:

1. Ultra short: <1 min, e.g. neck shot
2. Short: ±5 min, e.g. hanging
3. Long: ±15 min to 1 h, e.g. intoxication
4. Multiple hours/days, e.g. infectious complications after trauma

Statistical analysis

Statistical analysis was performed, using the SPSS programme 15.0.1 for Windows. Only non-parametric tests were performed due to the low number of cases in the subgroups. Correlations were tested with the aid of a Spearman's rho correlation coefficient. Differences between more than two subgroups or between two subgroups were tested by means of Kruskal–Wallis test or a Mann–Whitney *U* test, respectively. A p value ≤ 0.05 was considered statistically significant. The results are expressed as medians (±inter-quartile range (IQR)) or means (±SD). The ICC was used to evaluate the inter-observer variance; an ICC value of 1 is considered as a full agreement.

Results

IHC staining patterns

Four different staining patterns (see Fig. 1) were detected while evaluating the tissue slides: on the one hand, neurons with ubiquitin-immunoreactivity solely in the nucleus, solely in the cytoplasm or both in the nucleus and in the cytoplasm; on the other hand, neurons in which the ubiquitin-immunoreactivity was either absent or reduced to background staining (see Fig. 2). As internal positive controls, ubiquitin-immunoreactivity was also evaluated in the corpora amylacea and the ependymal cells. All negative controls showed no ubiquitin-immunoreactivity.

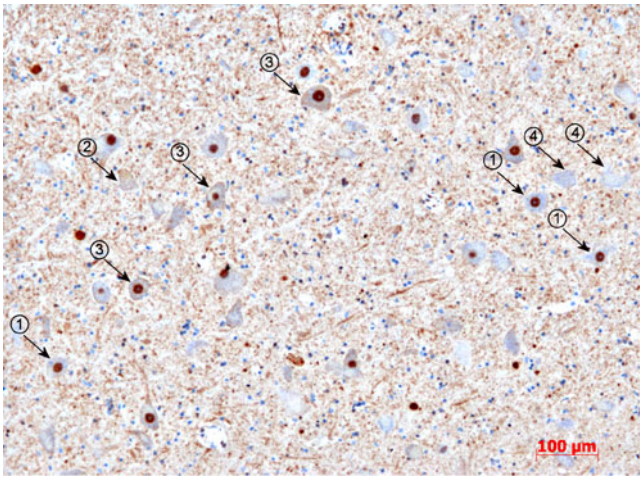


Fig. 1 Microscope photograph of the locus coeruleus (case 9, 1:600 dilution polyclonal rabbit anti-ubiquitin antibody, ×100 microscope magnification) displaying the four different types of ubiquitin-immunoreactivity: *1* ubiquitin solely in the nucleus, *2* ubiquitin solely in the cytoplasm, *3* ubiquitin both in the nucleus and cytoplasm and *4* no ubiquitin-immunoreactivity

Counting the neurons

The distribution of the total number of neurons in the locus coeruleus per square micrometer and the distribution of the total number of IHC positive neurons per square micrometer for each subgroup are presented in Table 2.

Most diversity in numbers is seen in the subdivision of the number of positive neurons, while the total number of IHC positive neurons remains approximately constant between the different researchers (ICC, 0.899). Therefore, we decided to use the total number of IHC positive neurons, instead of the subdivisions.

Descriptive statistics of the medico-legal study group

Locus coeruleus specimens were obtained from various forensic autopsy cases (n=48) (30 males (62.5%) and 18 females (37.5%)), aged from 2 to 87 years (mean, 43.3± 20.83 years; median, 41.5±24.00 years). Thirty-one (64.6%) of them had no reanimation, whereas for 17 (35.4%) victims, a reanimation attempt was performed. The PMI ranged from 11 to 762 h (mean, 82.0±120.28 h; median, 48.0±49.00 h).

The cause of death was subdivided into eight groups: mechanical asphyxia (n=8), intoxication with sedative drugs (n=4), intoxication without sedative drugs (n=4), blunt (poly) trauma with brain damage (n=5), blunt (poly) trauma without brain damage (n=10), cut, stab and gunshot

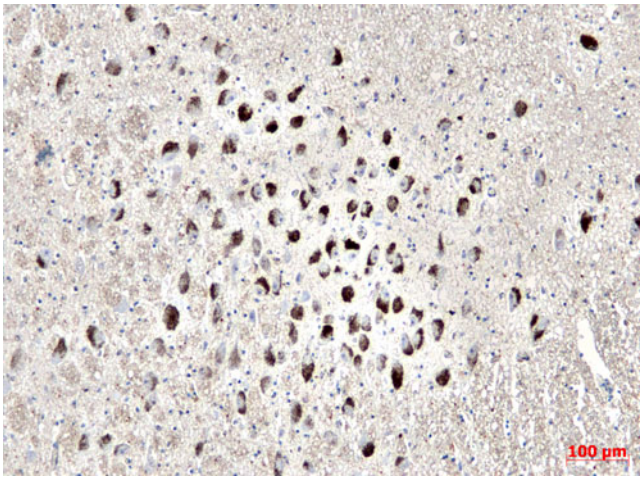


Fig. 2 Microscope photograph of the locus coeruleus (case 18, 1:600 dilution polyclonal rabbit anti-ubiquitin antibody, ×100 microscope magnification): displaying neurons with absent ubiquitin expression. NB: melanin pigment visible

wounds (n=7), hypothermia (n=1) and natural death (n=9). Brain damage, such as internal bleeding, was not present in the natural death cases.

The manner of death was subdivided into five groups: accidental (n=8), criminal (n=21), natural (n=10), suicidal (n=6) and undetermined (n=3).

The length of agony was classified in four groups: ultrashort (n=7), short (n=15), long (n=6) and multiple hours/days (n=20).

Table 3 presents the full case profiles.

Correlations and differences

The age and total number of neurons per square micrometer as well as the total number of IHC positive neurons per square micrometer did not show a significant correlation. No significant correlation was observed between the ubiquitin-immunoreactivity (the number of positive neurons per square micrometer) and the PMI. No significant differences were observed in the total number of neurons per square micrometer with respect to the length of agony nor in the number of IHC positive neurons per square micrometer with respect to the manner of death, the gender and the reanimation.

Figures 3 and 4 show the number of IHC positive neurons per square micrometer with respect to the cause of death and the length of agony, respectively. In addition, for each subgroup the median±IQR is displayed.

'Hypothermia' as a cause of death was excluded due to the small sample size (n=1). A significant difference in the number of IHC positive neurons per square micrometer with respect to the cause of death was noticed (p=0.038).

Considering the various causes of death, six significant differences were found: mechanical asphyxia versus intoxication without sedatives (p=0.016), intoxication with sedatives versus intoxication without sedatives (p=0.029), intoxication without sedatives versus blunt (poly) trauma with brain damage (p=0.016), intoxication without sedatives versus blunt (poly) trauma without brain damage (p=0.008), intoxication without sedatives versus cut, stab and gunshot wounds (p=0.006) and blunt (poly) trauma without brain damage versus cut, stab and gunshot wounds (p=0.043).

A significant difference in the number of IHC positive neurons per square micrometer with respect to the length of agony was observed (p=0.010).

More specifically to the length of agony, three significant differences in subgroups were revealed: ultrashort versus short (p=0.004), ultrashort versus long (p=0.035) and short versus multiple hours/days (p=0.030).

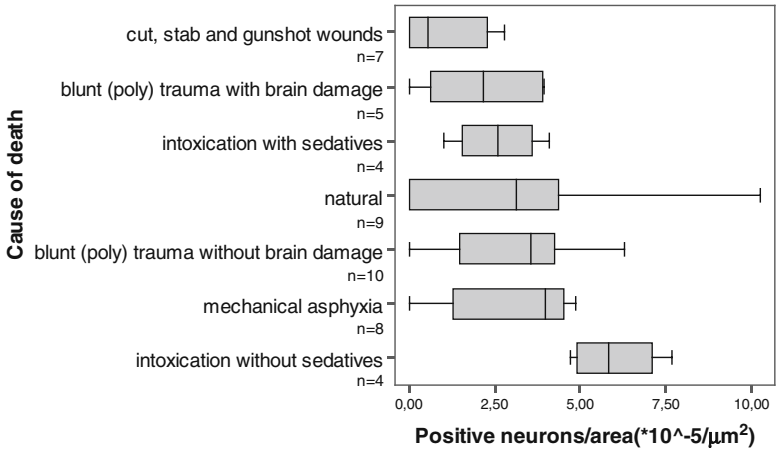
Discussion

An estimation of the survival time as well as the physical and mental load during the agonal phase is an important consideration to address in current forensic practice. We aimed to find a relationship between the amount of neuronal ubiquitin in the locus coeruleus and the agonal stress by means of an IHC technique.

Considering the IHC staining pattern (see Fig. 1), most of the neurons demonstrate ubiquitin-immunoreactivity in the nucleus. This is also confirmed by the literature, since it

Table 2 The distribution of the IHC staining patterns

IHC staining pattern	Range (neurons × 10^{-5}/µm^2)	Mean (neurons × 10^{-5}/µm^2)	SD (neurons × 10^{-5}/µm^2)	Median (neurons × 10^{-5}/µm^2)	IQR (neurons × 10^{-5}/µm^2)
Total neurons	4.62–30.52	11.91	4.641	10.93	5.25
Total positive neurons	0.00–10.28	2.86	2.327	2.95	3.61
Solely positive in nucleus	0.00–9.49	2.20	1.910	2.08	2.67
Solely positive in cytoplasm	0.00–3.17	0.45	0.724	0.17	0.64
Positive both in nucleus and cytoplasm	0.00–2.04	0.21	0.473	0.00	0.18



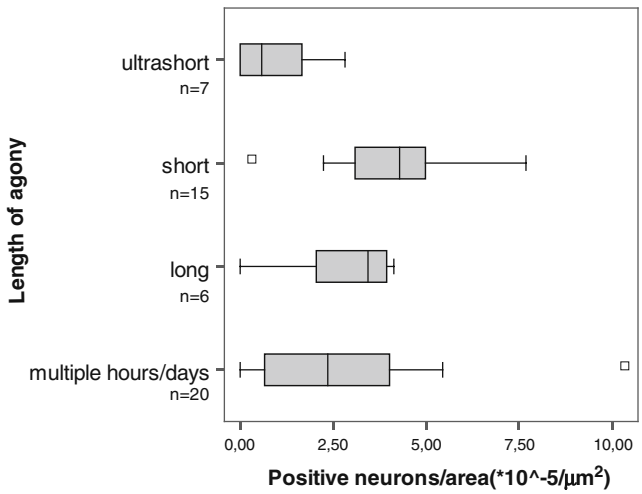
Cause of death	median ± IQR (neurons*10^{-5}/µm^2)
cut, stab and gunshot wounds	0,57 ± 2,29
Blunt (poly) trauma with brain damage	2,16 ± 3,61
Intoxication with sedatives	2,58 ± 2,58
Natural	3,15 ± 4,91
Blunt (poly) trauma without brain damage	3,55 ± 2,86
Mechanical asphyxia	3,99 ± 4,07
Intoxication without sedatives	5,83 ± 2,59

Fig. 3 Boxplots displaying the number of positive neurons per square micrometer (10^{-5}/µm^2) with respect to the cause of death (n=47, exclusion of hypothermia case), results expressed as median±IQR. The agonal distribution for each cause of death can be retrieved in Table 3

is believed that the ubiquitin can migrate from the cytoplasm to the nucleus [13–15] and presumably bind to the histones [22]. One could assume that the longer the agony lasts, the more neurons will show ubiquitin-immunoreactivity in the nucleus and less in the cytoplasm.

The different causes of death were classified into subgroups (see Fig. 3; exclusion of hypothermia case). The significant difference in ubiquitin-immunoreactivity between the 'cut, stab and gunshot wounds' and both the 'intoxication without sedatives' and the 'blunt (poly) trauma without brain damage' can point to the conclusion of a significantly lower ubiquitin-immunoreactivity in cut, stab and gunshot wounds. This lower IHC reactivity may suggest a shorter agony and thus less agonal stress.

The significantly higher ubiquitin-immunoreactivity in 'intoxication without sedatives' can be due to a significantly more pronounced agonal stress. The absence of a significant difference in immunoreactivity with the 'natural' deaths can be due to the varying length of the agonal phase in this subgroup. This varying length can be caused by the occurrence of various mechanisms of death (e.g. cardiac arrhythmia versus multiple organ failure). Remarkably, a significantly lower ubiquitin-immunoreactivity is observed in 'intoxication with sedatives' compared to 'intoxication without sedatives'. This can indicate less agonal stress during the 'intoxication with sedatives' due to the inherent properties of these drugs (sedative, relaxing effect). The significantly higher ubiquitin-immunoreactivity observed in intoxication without sedatives might also be induced by the neuronal toxicity of the toxicants CO (carbon monoxide) and cocaine. The neurotoxic effects of CO were researched by Tofighi et al. Their results suggested that CO is able to



Length of agony	median ± IQR (neurons*10^{-5}/µm^2)
Ultrashort	0,57 ± 1,90
Short	4,28 ± 2,78
Long	3,42 ± 2,43
Multiple hours / days	2,35 ± 3,43

Fig. 4 Boxplots displaying the number of positive neurons per square micrometer (10^{-5}/µm^2) with respect to the length of agony (n=48), results expressed as median±IQR. The distribution of the cause of death for each length of agony can be retrieved in Table 3

induce cell death by apoptosis [23]. This can point to a direct neuronal toxicity with an obviously higher neuronal ubiquitin immunopositivity. However, Quan et al. could not establish a correlation of the ubiquitin-positivity in the pigmented neurons of the substantia nigra with the blood carboxyhaemoglobin (COHb) level in fatal CO poisoning cases [13]. Since this is in opposition to our results, a higher susceptibility of the locus coeruleus to the agonal stress may be assumed. CO exposure can manifest itself in, e.g. headache, dizziness, weakness, hypoxia and tachycardia, which might all increase the agonal stress. Cocaine is able to augment the cellular stress, which can be confirmed by the up-regulation of the heat shock protein (HSP 72 kDa) expression, resulting in neuronal and glial cell damages [24]. However, it is not possible to discriminate whether the agonal stress and/or the neurotoxicity of the toxicants might be responsible for the ubiquitin immunopositivity. In fact, it cannot be excluded that both components might affect each other and influence the global stress reaction of the human being.

No significant differences were observed between 'cut, stab and gunshot wounds' death and resp. 'blunt (poly) trauma with brain damage', 'intoxication with sedatives', 'natural' death and 'mechanical asphyxia' which can also be explained by the varying length of agonal stress within these subgroups. Quan et al. described significant differences in the percentages of the nuclear ubiquitin-immunoreactivity in the subgroup 'mechanical asphyxia' [14]: This can point to the assumption that a fatal severe stress on the central nervous system can induce an intranuclear ubiquitin-immunoreactivity of the pigmented substantia nigra neurons.

Our study group is classified into four subgroups according to the length of agony (see Fig. 4). The determination of the length of agony, during the evaluation of the medico-legal files, was not always an obvious decision since it is determined by multiple parameters, e.g. the cause and mechanism of death, the histology and weight of various organs. However, taking into account multiple parameters and by using the combined but independent opinion of three researchers, we believe that any possible misclassification has been minimalised.

The most significant statistical difference is seen in the ubiquitin-immunoreactivity per square micrometer between the ultrashort and short agony period. This can lead to the assumption that the ubiquitin production or redistribution [15] in the first agonal minute (ultrashort agony) starts slowly, reaching a maximum at around 5 min into the agonal phase. During the next hour (long agony), the ubiquitin-immunoreactivity remains high (slight decreasing tendency), still showing a significant difference to the ubiquitin-immunoreactivity in the ultrashort agony. After more than 1 h of agony (multiple hours/days), the ubiquitin is elevated (a further slight decreasing tendency over the long agony). However, there is no significant difference between the 'multiple hours/days' and the 'ultrashort' agony, but this could be due to the wider spread of results in the 'multiple hours/days' agony. A significant difference is however seen between the ubiquitin-immunoreactivity of the 'short' and the 'multiple hours/days' agony, indicating a statistically significant decline in the ubiquitin-immunoreactivity in the multiple hours/days agony. This decline in positivity can be caused by the presence of ubiquitin-negative victims (cases 10, 21, 35 and 43) in combination with the wider range of results. The reason for the absence of obvious ubiquitin-immunoreactivity can be explained by the condition (brain death) of the victim during the very long agonal phase (multiple hours/days). It is not known if unconsciousness or a non-reactive coma, for example, can contribute to the agonal stress. At present, it is scientifically not possible to fully quantify the physical and mental stress. It can be presumed that the ubiquitin detection in the locus coeruleus can indicate both kinds of stress, since the locus has neuronal projections to several areas in the brain [25, 26]. The locus coeruleus plays a role in the stress response in the hypothalamo-pituitary-adrenal axis [27] and is thought to act as an alarm system [1]. The ubiquitin-immunoreactivity in the substantia nigra, on the other hand, might be a marker for the stress, resulting from the physical activity before death [13, 14].

The increase of ubiquitin-positive neurons may be the result of an overactivity of the noradrenergic neurons in the locus coeruleus during the agonal phase leading to an increased ubiquitin synthesis or redistribution.

A careful distinction should be made between the ultrashort agony and the longer ones on the basis of the ubiquitin-immunoreactivity, although some victims suffering from a longer agony show no obvious ubiquitin-immunoreactivity. Ubiquitin-immunoreactivity reduced to background should be cautiously evaluated, paying particular attention to, inter alia, the conscious state of the victim during the agony and the PMI. Particularly brain death must be borne in mind.

Questions were raised concerning the influence of narcotics, sedatives and other consciousness-reducing substances on reducing the agonal stress response. Indeed, when considering intoxications with (n=4) versus without (n=4) sedatives, a significant difference was observed, even in this small sample (p=0.029). This is in accordance with Quan et al. who found a higher neuronal ubiquitin-immunoreactivity in the midbrain periaqueductal grey matter in an acute methamphetamine (stimulant) fatality [28].

These preliminary results indicate significant differences in the number of ubiquitin-positive neurons in the locus coeruleus in case of a pronounced and extended death

struggle. This is in line with the cause of death and the presumed agonal period by means of the medico-legal reports. Although a careful distinction can be made between the ultrashort and the longer agonal periods, no clear distinction can be made, by means of this study, between the short agony, the long agony and multiple hours/days on the basis of the ubiquitin-immunoreactivity. A more extended research, combining various methods, should make it possible to raise the significancy, to notice any possible differences or correlations with respect to the length of the agony and to give an answer to this important topic. According to the study of Wilke et al. [29], the adrenalin/noradrenalin quotient in the various body fluids can contribute to a more efficacious distinction and correlation. Indeed, agonal stress is a complex intermingling of at least the length of agony and intensity of the agony. Furthermore, the intensity of physical pain, mental suffering and cellular distress all contribute to the global stress. In this study, instead of the agonal stress, the expression 'cellular distress' could be more appropriate. To discern the components of the global stress, the incorporation of ubiquitin activity in other cerebral nuclei and biochemical indicators (such as cortisol, lactate, catecholamines [30], chromogranin A [31] or adrenocorticotropic hormone [32]) could induce a better evaluation of the agonal stress.

Acknowledgement The authors like to thank Dr. Ellen Deschepper for the statistical advice.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Hendelman WJ (2000) Atlas of functional neuroanatomy. CRC, Boca Raton
2. Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53:865–871
3. Gos T, Hauser R (1996) Evaluation of the emotional state shortly before death—science-fiction or a new challenge? Int J Legal Med 108:327–328
4. Longstaff A (2005) Neuroscience. Taylor & Francis, New York
5. Fornace AJ, Alamo I, Hollander MC, Lamoreaux E (1989) Ubiquitin messenger-Rna is a major stress-induced transcript in mammalian-cells. Nucleic Acids Res 17:1215–1230
6. Finley D, Ozkaynak E, Varshavsky A (1987) The yeast polyubiquitin gene is essential for resistance to high-temperatures, starvation, and other stresses. Cell 48:1035–1046
7. Ciechanover A, Schwartz AL (1998) The ubiquitin–proteasome pathway: the complexity and myriad functions of proteins death. Proc Natl Acad Sci USA 95:2727–2730
8. Glickman MH, Ciechanover A (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428
9. Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67:425–479
10. Ishikawa T, Zhu BL, Li DR, Zhao D, Michiue T, Maeda H (2007) Immunohistochemical investigation of ubiquitin and myoglobin in the kidney in medicolegal autopsy cases. Forensic Sci Int 171:136–141
11. Lodish H, Berk A, Matsudaira P (2004) Molecular cell biology. Freeman, New York
12. Alves-Rodrigues A, Gregori L, Figueiredo-Pereira ME (1998) Ubiquitin, cellular inclusions and their role in neurodegeneration. Trends Neurosci 21:516–520
13. Quan L, Zhu BL, Oritani S, Ishida K, Fujita MQ, Maeda H (2001) Intranuclear ubiquitin immunoreactivity in the pigmented neurons of the substantia nigra in fire fatalities. Int J Legal Med 114:310–315
14. Quan L, Zhu BL, Ishida K, Oritani S, Taniguchi M, Fujita MQ, Maeda H (2001) Intranuclear ubiquitin-immunoreactivity of the pigmented neurons of the substantia nigra in fatal acute mechanical asphyxiation and drowning. Int J Legal Med 115:6–11
15. Pagano M (1997) Cell cycle regulation by the ubiquitin pathway. FASEB J 11:1067–1075
16. Dickson DW, Wertkin A, Kress Y, Ksiezak-Reding H, Yen SH (1990) Ubiquitin immunoreactive structures in normal human brains. Distribution and developmental aspects. Lab Invest 63:87–99
17. Quan L, Ishikawa T, Michiue T, Li DR, Zhao D, Zhu BL, Maeda H (2005) Quantitative analysis of ubiquitin-immunoreactivity in the midbrain periaqueductal gray matter with regard to the causes of death in forensic autopsy. Leg Med (Tokyo) 7:151–156
18. Moss A, Blackburn-Munro G, Garry EM, Blakemore JA, Dickinson T, Rosie R, Mitchell R, Fleetwood-Walker SM (2002) A role of the ubiquitin–proteasome system in neuropathic pain. J Neurosci 22:1363–1372
19. Madea B, Dettmeyer R, Schmidt P (2003) In: Madea B (ed) Praxis Rechtsmedizin: Befunderhebung, Rekonstruktion, Begutachtung. Springer, Berlin, pp 17–18
20. Dako. CSA II Biotin-free Tyramide Signal Amplification System, code K1497. For use with mouse primary antibodies
21. Cisse S, Perry G, Lacosteroyal G, Cabana T, Gauvreau D (1993) Immunochemical identification of ubiquitin and heat-shock proteins in corpora-amylacea from normal aged and Alzheimers-disease brains. Acta Neuropathol 85:233–240
22. Finley D, Varshavsky A (1985) The ubiquitin system—functions and mechanisms. Trends Biochem Sci 10:343–347
23. Tofighi R, Tillmark N, Dare E, Aberg AM, Larsson JE, Ceccatelli S (2006) Hypoxia-independent apoptosis in neural cells exposed to carbon monoxide in vitro. Brain Res 1098:1–8
24. Sharma HS, Muresanu D, Sharma A, Patnaik R (2009) Cocaine-induced breakdown of the blood-brain barrier and neurotoxicity. Int Rev Neurobiol 88:297–334
25. Nolte J (1993) The human brain: an introduction to its functional anatomy. Mosby-Year Book, St Louis
26. Moore RY, Bloom FE (1979) Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annu Rev Neurosci 2:113–168
27. Nieuwenhuys R, Voogd J, van Huijzen C (2007) The human central nervous system. Springer, New York
28. Quan L, Ishikawa T, Michiue T, Li DR, Zhao D, Oritani S, Zhu BL, Maeda H (2005) Ubiquitin-immunoreactive structures in the midbrain of methamphetamine abusers. Leg Med (Tokyo) 7:144–150
29. Wilke N, Janssen H, Fahrenhorst C, Hecker H, Manns MP, Brabant EG, Troeger HD, Breitmeier D (2007) Postmortem determination of concentrations of stress hormones in various body fluids—is there a dependency between adrenaline/noradren-

aline quotient, cause of death and agony time? Int J Legal Med 121:385–394
30. Zhu BL, Ishikawa T, Michiue T, Li DR, Zhao D, Quan L, Oritani S, Bessho Y, Maeda H (2007) Postmortem serum catecholamine levels in relation to the cause of death. Forensic Sci Int 173:122–129
31. Yoshida C, Ishikawa T, Michiue T, Quan L, Maeda H (2009) Postmortem biochemistry and immunohistochemistry of chromogranin A as a stress marker with special regard to fatal hypothermia and hyperthermia. Int J Legal Med. doi:10.1007/s00414-009-0374-3
32. Ishikawa T, Quan L, Li DR, Zhao D, Michiue T, Hamel M, Maeda H (2008) Postmortem biochemistry and immunohistochemistry of adrenocorticotropic hormone with special regard to fatal hypothermia. Forensic Sci Int 179:147–151