

1 **EFFECT OF HANDLING ON NEUROTRANSMITTER PROFILE IN PIG BRAIN**  
2 **ACCORDING TO FEAR RELATED BEHAVIOUR**

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23 **ABSTRACT**

24

25 Chemical neurotransmitters (NT) are principal actors in all neuronal networks of animals. The  
26 central nervous system plays an important role in stress susceptibility and organizes the  
27 response to a stressful situation through the interaction of the dopaminergic and the  
28 serotonergic pathways, leading to the activation of the hypothalamus-pituitary-adrenal axis  
29 (HPA). This study was designed to investigate: a) the effects of stressful handling of pigs at  
30 the slaughterhouse on the neurotransmitter profile in four brain areas: amygdala, prefrontal  
31 cortex (PFC), hippocampus and hypothalamus, and b) whether the alterations in the brain NT  
32 profile after stressful handling were associated with fear, determined by the tonic immobility  
33 (TI) test. In the first place, the characterization of the NT profile allowed to distinguish the  
34 four brain areas in a principal component analysis. The most crucial pathway involved in the  
35 reaction of pigs to a stressful handling was the serotonergic system, and changes were  
36 observed in the amygdala with a decrease in serotonin (5-HT) and total indoleamines, and in  
37 the hippocampus, where this pathway was activated. Fearful and non-fearful pigs did not  
38 show significant differences in their NT profile in control conditions, but when subjected to a  
39 stressful handling in the slaughterhouse, fearful animals showed a significant variation in the  
40 serotonin pathway and, in a lesser extent, the dopamine (DA) pathway. In conclusion, the  
41 existence of an underlying biological trait - possibly fearfulness - may be involved in the pig's  
42 response toward stressful challenges, and the serotonergic system seems to play a central role  
43 in this response.

44 **Keywords:** brain, dopamine, pig, serotonin, stress, tonic immobility

## 45 **1. Introduction**

46 Chemical neurotransmitters (NT) are principal actors in all neuronal operations. The  
47 noradrenergic, dopaminergic and the serotonergic pathways are the most important and well  
48 characterized systems underlying the response to stress, fear and reward, among others. The  
49 central nervous system controls the action of endocrine glands through the release of  
50 catecholamines, indoleamines and other transmitters which can be excitatory or inhibitory  
51 mediators [1]. Amygdala, hippocampus and prefrontal cortex (PFC) are recognized to play a  
52 role in the stress response organization. In these structures, stressors produce changes in  
53 extracellular concentrations of different NTs leading to activation and modulation of  
54 processes to cope with stress. These areas have an indirect output to the hypothalamus, which  
55 acts modulating the final stress response through the sympathetic nervous system and the  
56 activation of the hypothalamic-pituitary-adrenal (HPA) axis [2]. Therefore, the stress response  
57 involves not only the activity of these specific brain areas, but also the interaction among  
58 those areas through neuromodulators, especially catecholamines (noradrenaline (NA),  
59 dopamine (DA)) and the indoleamine serotonin (5-HT). DA is metabolized to homovanillic  
60 acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC), whereas 5-HT is metabolized to  
61 5-hydroxyindoleacetic acid (5-HIAA) [1]. Determining the ratios between the amine and its  
62 metabolites can indicate the turnover rate [3].

63 Tonic immobility (TI) is a well-established test to evaluate the fear response in a wide range  
64 of vertebrates and invertebrates [4,5]. Long duration of TI is generally considered as an  
65 indication for high levels of fearfulness associating tonic immobility with emotional  
66 components like fear or anxiety [6] and with a fear-related phenotype [7]. In pigs, the TI test  
67 has shown to be consistent with other behavioural tests carried out at different ages assessing  
68 fear, aggressiveness and behavioural strategies in front of a stressful situation, thus indicating  
69 that it may be related to individual personality characteristics [4,8–15]. A positive relationship

70 has been reported between TI scores and lean meat percentage, and a genetic background has  
71 been suggested [8,9]. Furthermore, the fear-related behaviour is closely associated with the  
72 stress response regulated by the HPA axis [7,16].

73 There are several stressors widely recognized and studied in pigs, such as handling, mixing,  
74 transport and slaughter [17]. One of the main consequences of pre-slaughter stress is the  
75 production of pale, soft and exudative (PSE) meat, leading to an organoleptic and economic  
76 cost [18]. In the literature, changes in brain NT profiles in genetically stress-susceptible pigs  
77 have been reported [19]. Immobilization of pigs produces changes in hypothalamic and/or  
78 hippocampal bioamine levels, suggesting an important role of these regions in the  
79 responsiveness of the pig to acute stress conditions [3,20]. Furthermore, the involvement of  
80 central nervous system NT in aggressiveness and dominance has also been studied [21–24].  
81 However, changes in brain NT related to standard or commercial stress conditions at slaughter  
82 and the fear-related behaviour have been rarely studied in pigs [25].

83 In the present study, we have first characterized the NT profile of catecholamines and  
84 indoleamines in four different brain areas of the pig involved in the stress and fear response:  
85 amygdala, PFC, hippocampus and hypothalamus. Secondly, we have analysed the changes in  
86 NT profile in pigs subjected to stress at slaughter classified according to a fear-related  
87 phenotype.

## 88 **2. Materials and Methods**

### 89 **2.1. Animals, housing conditions, general procedure and ethical statement**

90 This study was carried out at the IRTA-Monells experimental farm (Monells, Spain). Ninety-  
91 two male piglets were randomly allocated in 10 housing groups of 10-12 piglets each in the  
92 pre-control building at 3 weeks of age (mean  $\pm$  SE: 5.85  $\pm$  0.166 Kg). All piglets came from

93 the same commercial farm and were crosses of Large White × Landrace Halothane gene -  
94 RYR(1)- free (NN) sows with Pietrain heterozygous (Nn) terminal sire. At 4 weeks of age, all  
95 piglets were subjected to a TI test in order to select a total of 36 piglets (18 positive to TI and  
96 18 negative to TI, see 2.2). At 8 weeks of age, pigs were moved to the control building and  
97 randomly allocated in four groups of nine.

98 Each group was housed in slatted pens (5 m x 2.70 m) under natural light conditions at a  
99 constant environmental temperature of  $22 \pm 3$  °C. Each pen was provided with one steel  
100 drinker bowl (15 cm x 16 cm) connected to a nipple and a concrete feeder (58 cm x 34 cm)  
101 with 4 feeding places. Pigs had water and food *ad libitum*. The pigs were inspected daily and  
102 no health problems were observed during the experimental period. The study was approved  
103 by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

## 104 **2.2. Tonic immobility test**

105 Piglets were subjected to a TI test adapted from Erhard et al. [10] and de Sevilla et al. [8]. An  
106 experimenter restrained individually each piglet in a dorsal decubitus position using a V-  
107 shaped wooden restrain (50 cm long and with an 80° angle). Another experimenter placed a  
108 small bag (15 cm x 20 cm and weighing 500 g) over the piglet's throat with one hand, while  
109 carefully holding the hind legs with the other until the animal remained immobile. Only one  
110 induction was performed and the time between the experimenter's hands were removed from  
111 the animal's hind legs and the time that the piglet tried to turn was recorded. If the piglet did  
112 not try to turn within 3 min, the trial finalized, and the time of 180 s was assigned to this  
113 piglet. Otherwise, piglets that did not show the immobility response because they struggled  
114 while they were being placed onto the V-shaped wooden restrain were assigned a time of 0 s.  
115 The 18 piglets with the lowest time (less than or equal to 10 s) to try to turn were chosen and  
116 classified as negative to TI test and the 18 piglets with the highest time (equal to or more than

117 54 s) to try to turn were also selected and classified as positive to TI test. An outline of the  
118 experimental design and the distribution of TI negative and TI positive animals is shown in  
119 Figure 1.

### 120 **2.3. Housing and slaughtering conditions.**

121 Animals aged 24 weeks were fasted 8 h before being transported from the experimental farm  
122 to the experimental slaughterhouse (1.2 km of distance). “Control” and “stress” conditions  
123 included different management during unloading, lairage and conduction to the stunning area.  
124 During the unloading, the pigs of two housing pens (9 TI-negative, 9 TI-positive) were  
125 subjected to stress by noise, human presence and rough handling (simulating commercial  
126 conditions) whereas the pigs of the other two housing pens (9 TI-negative, 9 TI-positive) were  
127 handled very calmly allowing the time need for the animals to go ahead by themselves. Pigs  
128 were located in the lairage pens for an hour, the 18 animals under stressful conditions were  
129 mixed between the two housing groups, whereas the 18 animals under control conditions  
130 remained separated maintaining the housing groups. The management during the conduction  
131 of the pigs to the stunning area was similar to the management during unloading (control or  
132 stress). The total length of the procedure was approximately 90 min. Animals were stunned in  
133 groups of two by exposure to 90 % CO<sub>2</sub> at atmospheric air for 3 min and exsanguinated  
134 afterwards.

### 135 **2.4. Tissue sampling and neurotransmitter quantification.**

136 Immediately after the slaughter ( $\approx$ 5 min) the skull was opened. The brain was removed and  
137 tissue samples from the selected brain structures (amygdala, PFC, hippocampus and  
138 hypothalamus) were excised, collected as quickly as possible (within 90 s) in liquid N<sub>2</sub> and  
139 kept frozen at -80°C, until NT analysis according to a procedure adapted from Sabrià et al.  
140 [26]. Samples were weighted and homogenized (1:10 w/v) in ice-cold 0.25 M perchloric acid

141 containing 0.1 M Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> and 0.25 M ethylenediaminetetraacetate (EDTA).  
142 Dihydroxybenzylamine (DHBA) and N $\omega$ -metil-5-hydroxytryptamine (N $\omega$ ) were added as  
143 internal standards for catecholamines and indoleamines, respectively. The mixtures were  
144 homogenized by sonication (Branson Digital Sonifier, model 250, Branson Ultrasonics Corp.,  
145 Danbury, CT) followed by centrifugation at 3000 g for 10 min at 4°C and the supernatants  
146 were kept frozen at -80°C. After centrifugation at 12000 g for 10 min at 4°C, the  
147 concentration of catecholamines (NA, DA, DOPAC and HVA) and indoleamines (5-HT and  
148 5-HIAA) were determined in 20  $\mu$ L aliquots using HPLC (Elite LaChrom, Merck, Hitachi,  
149 Japan) equipped with a Chromolith Rp-18e 100 x 4.6 mm column (Merck KgaA, Darmstadt,  
150 Germany) with electrochemical detection (ESA Coulochem II 5200, Bedford, MA). The  
151 mobile phase consisted of 0.5 M citrate buffer pH 2.8, 0.05 mM EDTA, 1.2 mM sodium octyl  
152 sulphate (SOS) and 1% acetonitrile. The applied voltage was set at 400 mV and the flow rate  
153 was 1 mL/min.

154 The chromatographic quantification of dopaminergic and serotonergic NTs showed a good  
155 precision, with coefficient of variation between-days and within-days lower than 4%.  
156 Linearity was evaluated between 2.5 – 80 pg/ $\mu$ l for 5-HT, 5-160 pg/ $\mu$ l for N $\omega$ , 5-240 pg/ $\mu$ l for  
157 HVA and 2.5-120 pg/ $\mu$ l for the rest of NTs. Coefficients of determination (R<sup>2</sup>) were  
158 calculated and found to be higher than 0.999 for all analytes. Limit of detection was between  
159 2.14 and 4.97 pg/ $\mu$ L and the limit of quantification was between 6.48 and 15.06 pg/ $\mu$ L for all  
160 the analytes. The internal controls (DHBA and N $\omega$ ) allowed the comparison between runs.

161 Total content of catecholaminergic and serotonergic pathways and ratios of DOPAC and  
162 HVA to DA, and 5-HIAA to 5-HT were estimated as a measure of DA and 5-HT turnover or  
163 rate metabolism in these brain regions.

## 164 **2.5. Statistical analysis**

165 The statistical analysis was carried out with the Statistical Analyses System (SAS V9.2;  
166 software SAS Institute Inc., Cary, NC; 2002-2008). The significance level was established at  
167  $P < 0.05$  and a tendency was considered at  $0.05 \leq P \leq 0.1$ . Descriptive data are presented with  
168 the means and the standard error (mean  $\pm$  SE).

169 Whenever possible, data was log transformed to correct the distribution and hence permit use  
170 of parametric statistics. Normality test of data and residuals was performed for each measure.  
171 Normally distributed measures were analyzed using the MIXED procedure of SAS with  
172 Tukey adjustment. Measures with Poisson or multinomial distributions were analyzed using  
173 the GENMOD procedure of SAS. In all models, each pig was introduced as the experimental  
174 unit, the fixed effects included were type of handling and immobility test and planned pair-  
175 wise comparisons with Bonferroni correction were performed.

176 **Factor analysis:** Interrelations among the seven neurotransmitters were included in a  
177 common factor analysis using principal component solution (PCA) to identify unobserved  
178 common factors that explain differences between regions. The criteria used to determine the  
179 number of factors to retain were: (a) eigenvalues  $> 1$  and (b) total variance accounted greater  
180 than 60 %. After the initial factor extraction, the matrix was orthogonally rotated (varimax  
181 method) to maintain factors independent and uncorrelated. Thus, each variable had a high  
182 loading (correlation coefficient between variables and factors) on a single factor and a small  
183 or moderate loading on other factors, using 0.5 loading in absolute value as cut-off point to  
184 accept a variable into a factor.

185 Each brain region of each pig obtained an individual score on each factor. Factor scores were  
186 normally distributed with a mean of zero.

### 187 **3. Results**



188 **3.1. Tonic Immobility test**

189 The mean time of the 92 piglets to turn was  $34.80 \pm 3.77$  s. Five of the 92 piglets (5.43 %) did  
190 not show immobility response therefore they were classified as negative to TI. Three of the 92  
191 animals (3.26 %) did not turn during the 3 min of the test and were classified as positive to TI.  
192 Since this was not enough to build groups with the required sample size, the 18 individuals  
193 with the most extreme behaviours were chosen. The mean time of the animals negative to TI  
194 and positive to TI was  $5.00 \pm 0.93$  s and  $93.05 \pm 10.22$  s, respectively, thus both groups were  
195 considerably apart (Figure 1). Long duration of TI is considered as an indication for high  
196 levels of fearfulness, thus negative pigs to TI were classified as non-fearful animals and  
197 positive pigs as fearful animals

198 **3.2. Levels of brain amines and their metabolites in amygdala, PFC, hippocampus**  
199 **and hypothalamus.**

200 Table 1 shows the regional distribution of catecholamines and indoleamines in the four brain  
201 regions. Highest concentrations of NA were found in the hypothalamus. The concentration of  
202 DA and its metabolites DOPAC and HVA were found to be highest in the amygdala and  
203 hypothalamus. The ratio DOPAC/DA and HVA/DA was highest in the PFC.

204 Regarding to indoleamines, the highest concentration of 5-HT was found in the amygdala and  
205 hypothalamus, whereas the ratio 5-HIAA/5-HT was similar in all structures.

206 Principal Component analysis (PCA) reduced the seven variables (NA, L-DOPA, DOPAC,  
207 DA, HVA, 5-HIAA and 5-HT) to 2 common factors or principal components explaining 92.11  
208 % of the variance. The eigenvalues, the individual and cumulative percentage accounted and  
209 the varimax rotated factor loadings for each variable are shown in Table 2. Brain regions with

210 a high score for factor 1 (PC1) had high levels of DOPAC, DA, HVA, 5-HIAA and 5-HT; and  
211 brain regions with a high score for factor 2 (PC2) had high levels of NA and L-DOPA.

212 The PCA score plot showed that the pattern of NTs was able to readily differentiate all four  
213 brain areas (Figure 2).

### 214 **3.3. Influence of handling stress at slaughter on brain NTs.**

215 The concentrations of brain monoamines in the amygdala, PFC, hippocampus and  
216 hypothalamus are presented in Table 3.

217 The handling stress group presented lower concentration of 5-HT ( $P = 0.044$ ), HVA ( $P =$   
218  $0.028$ ) and a tendency for DA ( $P = 0.064$ ) in the amygdala. As a consequence, a decrease in  
219 total indole content was observed in this area ( $P = 0.043$ ). In the hippocampus, the  
220 concentration of 5-HIAA ( $P = 0.031$ ), 5-HT ( $P = 0.054$ , tendency) and total indole content ( $P$   
221  $= 0.024$ ) was found to be higher in animals exposed to handling stress. Catecholamine levels  
222 did not show difference between handling groups, but an increase in the ratio  
223 indole/catecholamine ( $P = 0.012$ ) was found in this area. In the hypothalamus, an increase in  
224 HVA ( $P = 0.017$ ) and in the sum of the metabolites DOPAC+HVA ( $P = 0.020$ ) was observed  
225 in stressed pigs. Finally, no difference in any monoamine and their metabolites was found in  
226 the PFC.

227 To find out whether the fearful individuals (TI positive) showed a higher response to a  
228 stressful situation in the slaughterhouse, pair-wise comparisons were performed within  
229 handling groups (Table 4). Indeed, there were no differences in the NT profile between fearful  
230 and non-fearful groups in the control situation. In contrast, significant differences were found  
231 between TI positive and TI negative groups when stressfully handled at the slaughterhouse.  
232 Fearful animals show an increase in total catecholamines ( $P = 0.047$ ), 5-HT ( $P = 0.030$ ) and a

233 tendency for total indoleamines ( $P = 0.063$ ) in the hippocampus and a tendency to increase L-  
234 DOPA ( $P = 0.090$ ) in the hypothalamus compared to non-fearful animals.

#### 235 **4. Discussion**

236 Following classical neurology, the neural pathways controlling response to stress, fear,  
237 aggression, emotion, decision-making and other behaviours are allocated in specific brain  
238 areas such as the amygdala, the hippocampus and the PFC [27–32]. They process sensory  
239 information to organize the autonomic response to stimuli from the environment or from  
240 internal cues and, in particular, these areas are involved in the control of stress and the  
241 regulation of the HPA axis [33]. Catecholaminergic (NA, DA and their metabolites), and  
242 serotonergic (5-HT and 5-HIAA) systems play a significant role in integrating the activity and  
243 interaction among those areas [1,34].

244 In this work, we have shown that, together with the hypothalamus, these areas are  
245 characterized by a particular pattern of NT that clearly discriminate the four regions. In the  
246 PCA, component 2 (high concentrations of NA and L-DOPA) characterized the hypothalamus  
247 versus the other three areas, whereas the first component (high concentrations of the other  
248 NTs) characterized the amygdala versus PFC and hippocampus. These two areas were the  
249 most similar in their NT profile.

250 All three NT systems (noradrenergic, dopaminergic and serotonergic) have an important role  
251 in the control of the stress reaction [34]. Although, historically, the noradrenergic system in  
252 the *locus coeruleus* has attracted much attention in the study of the stress response, the  
253 dopaminergic and the serotonergic systems have also been consistently implicated [35]. In  
254 particular, 5-HT has remarkable modulatory effects in almost all central nervous system  
255 integrative functions, such as stress, mood, anxiety and aggression [36] and it has been  
256 recognized as being directly related to stress and able to regulate the HPA axis by stimulating

257 CRH release in the paraventricular nucleus of the hypothalamus [37]. Marked changes in  
258 brain 5-HT turnover have been shown to occur in both rodents and humans upon activation of  
259 the HPA axis [38]. In particular, significant increases in the synthesis and release of 5-HT  
260 have been observed in various brain areas in response to different stressful conditions such as  
261 electrical foot shocks, cold environment, immobilization sessions, or tail pinches in rats [37].  
262 All these data strongly support the existence of reciprocal relationships between the 5-HT  
263 system and the HPA axis.

264 The results presented here support the central role of the serotonergic pathway in the  
265 regulation of the short term reaction to acute stress in pigs. The most remarkable change  
266 induced by stressful handling at the slaughterhouse is the alteration of the serotonergic system  
267 in the hippocampus and in the amygdala. There is a decrease in the serotonin pathway (5-HT  
268 and total indoleamines, and a tendency for 5-HIAA) in the amygdala after acute handling  
269 stress. It is known that under stress conditions the *locus coeruleus* activates stress pathways in  
270 the amygdala through noradrenergic projections. Likewise, the amygdala sends projections to  
271 the hypothalamus and brain stem, mediating the unconscious acute responses to danger and  
272 orchestrating the expression of behavioural and physiological responses (e.g. changes in heart  
273 rate, respiration and pupillary dilation) [35]. Thus, the amygdala and the hypothalamus are  
274 connected to innate (unconditioned) fear and may serve to enhance the state of arousal in  
275 order to adapt to challenging situations [39]. A decrease in 5-HT in the amygdala has been  
276 shown in rats subjected to forced swimming as a model of acute stress [34], although  
277 contradictory results have been reported that are probably explained by the existence of  
278 specific regions inside the amygdala with different functional roles [27].

279 The hippocampus is central to 5-HT function since it receives a dense projection of 5-HT  
280 fibres mainly from the *raphe nucleus* and it is rich in various 5-HT receptor types, being a  
281 mediator in the relationship of 5-HT with the HPA axis [32,37]. The increase of 5-HT

282 (tendency) as well as its metabolite 5-HIAA induced by stressful handling indicates that this  
283 NT is synthesized and rapidly metabolized. This is in agreement with the general idea that  
284 only inescapable, but not escapable, stresses produce an increase in extracellular 5-HT  
285 concentration in rat hippocampus [37,41]. In rodents, many studies have demonstrated that 5-  
286 HT release is increased in the hippocampus during several stress conditions, including  
287 immobilization [42], psychological stress [43], exposure to cats, tail pinch and forced  
288 swimming [44] and footshock [45,46].

289 As stated above, the amygdala sends the distress signal to the hypothalamus, where an  
290 increase in HVA and HVA+DOPAC is observed, indicating a higher rate of DA catabolism.  
291 This indicates that the DA system, and not only the NA system, is activated by stressful  
292 stimuli, as suggested by others [35]. No changes in NA were detected in the hypothalamus in  
293 the present work, in contrast to the reported decrease in NA in this region in pigs that showed  
294 distressed behaviour at the slaughterhouse [25] and to acute immobilization stress [3,20], but  
295 their approach was different from our experimental setting.

296 TI is a measure of fear and this fear-related behaviour is closely associated with the stress  
297 response regulated by the HPA [7,47,48]. Due to this relationship, we analysed the alteration  
298 in NTs associated to the response to the TI test. To get a more accurate analysis of the NT  
299 response to fear, we analyzed the response of the animals to control or stressful handling at  
300 the slaughterhouse depending on their TI classification. Fearfulness could be considered a  
301 basic feature of the temperament of each individual, that predisposes it to respond to a variety  
302 of potentially alarming challenges [49].

303 The objective was to establish differences between individuals classified as having a fear-  
304 related behaviour and those classified as non-fearful. Indeed, there were no significant  
305 differences between fearful and non-fearful animals in control conditions. In contrast, fearful

306 animals displayed important changes in the NT profile in the stressful situation. Again, the  
307 serotonergic pathway was mostly affected, especially in the hippocampus. The hippocampus  
308 and the serotonergic system have been previously related to fear behaviour in pigs [50] and  
309 chicken [51]. In pigs, Ursinus et al. [50] recently reported that hippocampal 5-HT is positively  
310 correlated with standing alert time (freezing) and inversely correlated with locomotion and  
311 exploration in pigs subjected to a novel object test. Since freezing is a sign of fear and  
312 explorative behaviours are generally thought to reflect a low level of fear or anxiety, it is  
313 concluded that hippocampal 5-HT increases in a fear condition [52]. The authors did not find  
314 any relationship between behaviour in the novel object test and 5-HT levels in the PFC or in  
315 the hypothalamus. In agreement with these authors, our results support the hypothesis that the  
316 relations between behaviour and measures of 5-HT in brain indicate an underlying personality  
317 trait and that individual differences in behaviour of animals during environmental challenges  
318 may covary with the animal's serotonergic system functioning. It is also interesting to note  
319 that in Ursinus's work, hippocampal 5-HT activity measured at 19 weeks of age in euthanized  
320 animals was related to behaviours observed during the novelty test at 11 weeks of age [50].  
321 Taken altogether, these results suggest that the hippocampus, but not other brain regions,  
322 might be involved in a putative personality measure in pigs related to the trait fearfulness, and  
323 that 5-HT would be the main neurotransmitter involved. In rats, a short lasting acute  
324 footshock session was able to induce a marked increase in 5-HT synaptic levels in the  
325 hippocampus as well as freezing and anxiety-related behaviours [46], and endogenous 5-HT  
326 seems to be responsible for the modulation of activity in the hippocampal pyramidal neurons  
327 linked to freezing behaviour [53]. Furthermore, mice with a genetic deletion of the serotonin  
328 1A receptor (5-HT<sub>1A</sub>R) have been shown to be more fearful in a number of behavioural  
329 conflict tests, confirming the important role of this neurotransmitter and this receptor in  
330 modulating anxiety [29,54]. Thus, the role of the hippocampus and the serotonergic system in

331 the fear-related responses to stressful challenges may be a general characteristic of animal  
332 species. Although our results indicate a principal role of 5-HT and the hippocampus, they also  
333 suggest the involvement of the catecholamine system, since total catecholamines are  
334 increased in this region.

335 It is interesting to speculate about the molecular changes that lead to variations in the NT  
336 concentration, taking into account that the methodological approach used in the present work  
337 measures the total amount of the NT. It is not possible in pigs to perform microdialysis  
338 experiments that would allow the direct measurement of extracellular NTs (presumably  
339 related to their presence at the synapsis) [55]. The mechanism involving NT release includes  
340 the synthesis of the NT by synthetic enzymes, their recruitment to vesicles and their release to  
341 the synaptic cleft [56]. Since we are measuring total content of NTs, rapid changes in total 5-  
342 HT concentration could be related to a change of tryptophan hydroxylase 2 activity (TPH2),  
343 the enzyme that catalyzes the rate-limiting step in serotonin biosynthesis in the brain [57].  
344 Current knowledge indicates that TPH2 is specifically transcribed in the somatodendritic  
345 segment of 5-HT neurons and a variable fraction of TPH2 mRNA is transported to terminal  
346 field [58]. A similar regulatory mechanism exist for tyrosine hydroxylase, the rate-limiting  
347 enzyme for the synthesis of catecholamines [59]. These enzymes can be rapidly transcribed in  
348 response to acute stress such as immobilization or other types of stress [27,60]. Other  
349 regulatory mechanisms that may be potentially involved are phosphorylation by protein  
350 kinase A (PKA) and the Ca<sup>2+</sup>/calmodulin dependent protein kinase II [61–63] and protein-  
351 protein interactions [59].

## 352 **Conclusions**

353 The most remarkable change induced by stressful handling is the alteration of the serotonergic  
354 system in the hippocampus and in the amygdala. There was no difference in neurotransmitter

355 profile between fearful and non-fearful pigs when confronted to a non-stressful handling at  
356 the slaughterhouse, but fearful animals did show more changes when subjected to stressful  
357 handling, concerning specially the serotonergic pathway in the hippocampus.

358 In conclusion, the existence of an underlying biological trait - possibly fearfulness - may be  
359 involved in pig's response toward stressful challenges, and the serotonergic system seems to  
360 be central to this response.

361



362 **Conflict of interest statement**

363

364 The authors declare that there is no conflict of interest associated with this manuscript.

365

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373

374 **Legends to the figures**

375

376 **Figure 1.** Outline of the experimental design and the distribution of TI negative and TI  
377 positive animals. Ninety-two pigs were subjected to the TI test with a maximum allowed time  
378 of 180 s. Three pigs did not turn (TI positive) and five moved immediately (time = 0 s, TI  
379 negative). The animals showing the most extreme responses in reaction time were selected to  
380 be included in the study (18 animals for each group).

381

382 **Figure 2.** Score plot from a principal component analysis showing the distribution of the four  
383 brain areas analysed (amygdala, PFC, hippocampus and hypothalamus) regarding their NT  
384 profile.

385

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- 553

Table 1. Concentration of neurotransmitters (ng/g tissue) in brain areas

	Amygdala		PFC		Hippocampus		Hypothalamus	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
NA	152.50	9.37	144.39	4.00	130.48	5.03	2007.19	83.77
L-DOPA	-	-	-	-	-	-	309.91	18.73
DOPAC	43.65	4.04	7.28	0.86	-	-	59.40	7.19
DA	359.69	24.42	19.95	1.31	27.52	2.63	302.27	15.05
HVA	280.27	18.39	61.45	4.93	-	-	295.61	22.09
Total Catecholamines	860.48	40.86	232.62	11.13	154.81	5.31	2911.25	103.77
5-HIAA	270.97	13.22	111.10	5.16	129.74	5.41	373.24	23.09
5-HT	917.38	47.35	252.71	10.79	276.56	9.83	995.47	51.79
Total Indoleamines	1188.35	58.29	363.81	14.66	406.30	13.65	1368.71	70.88
5- HIAA/5-HT	0.31	0.01	0.45	0.02	0.48	0.02	0.08	0.02
DOPAC/DA	0.11	0.01	0.37	0.04	-	-	0.19	0.02
HVA/DA	0.91	0.09	3.34	0.24	-	-	1.03	0.09
NA/DA	0.83	0.38	8.39	0.63	6.55	0.91	6.94	0.33
(DOPAC+HVA)/DA	0.97	0.04	3.83	0.27	-	-	1.28	0.12
DOPAC+HVA	330.88	19.84	67.41	5.81	-	-	335.77	26.89
Indoleamines/Catecholamines	1.452	0.05	1.79	0.12	2.68	0.126	0.48	0.02



Table 2. Eigenvalues, individual and cumulative proportion of the Correlation Matrix and loadings of neurotransmitters in varimax rotated factor matrix. Loadings equals or higher than 0.50 are highlighted to indicate the main attributes of the different principal components (PC).

	<b>PC1</b>	<b>PC2</b>
Eigenvalue	5.053	1.395
Individual Proportion	72.19%	19.92%
Cumulative Proportion	72.19%	92.11%
<b>Variables</b>		
NA	0.23	<b>0.97</b>
L-DOPA	0.22	<b>0.97</b>
DOPAC	<b>0.90</b>	0.28
DA	<b>0.95</b>	0.11
HVA	<b>0.93</b>	0.18
5-HIAA	<b>0.82</b>	0.46
5-HT	<b>0.91</b>	0.26

Table 3. Effects of stressful handling at the slaughterhouse on the neurotransmitter concentration (ng/g tissue) in amygdala, PFC, hippocampus and hypothalamus in pigs. \*  $P < 0.05$

	Variables	Handling at Slaughter				Effect
		CONTROL		STRESS		
		Mean	SE	Mean	SE	
<b>Amygdala</b>	NA	146.79	9.02	158.55	16.98	0.803
	DOPAC	47.38	5.79	39.17	5.54	0.319
	DA	403.52	32.34	313.27	34.20	<b>0.064</b>
	HVA	318.94	24.92	239.32	24.02	<b>0.028*</b>
	Total Catecholamines	916.63	61.81	793.10	47.24	0.134
	5-HIAA	292.67	18.31	248.00	17.99	<b>0.092</b>
	5-HT	1009.16	57.02	820.20	70.74	<b>0.044*</b>
	Total Indoleamines	1301.84	72.43	1068.20	84.90	<b>0.043*</b>
	5- HIAA/5-HT	0.29	0.01	0.32	0.02	0.336
	DOPAC/DA	0.11	0.01	0.11	0.01	0.917
	HVA/DA	0.83	0.06	1.00	0.18	0.614
	NA/DA	0.39	0.02	0.50	0.08	0.624
	(DOPAC+HVA)/DA	0.94	0.06	0.93	0.06	0.868
	DOPAC+HVA	366.32	28.82	291.00	24.11	<b>0.057</b>
	Indoleamines/Catecholamines	1.48	0.08	1.42	0.07	0.642
	<b>Prefrontal Cortex</b>	NA	139.11	5.77	150.80	5.10
DOPAC		7.61	1.24	6.87	1.21	0.830
DA		19.80	2.09	20.13	1.50	0.903
HVA		61.70	7.95	61.18	5.94	0.821
Total Catecholamines		227.58	14.36	239.94	18.22	0.595
5-HIAA		107.83	6.38	114.57	8.33	0.418
5-HT		255.24	15.10	250.03	15.89	0.835
Total Indoleamines		363.07	20.01	364.60	22.18	0.964
5- HIAA/5-HT		0.43	0.02	0.47	0.03	0.322
DOPAC/DA		0.36	0.05	0.37	0.06	0.949
HVA/DA		3.30	0.32	3.40	0.37	0.846
NA/DA		8.33	0.94	8.47	0.80	0.676
(DOPAC+HVA)/DA		3.74	0.34	3.96	0.43	0.679
DOPAC+HVA		68.41	9.04	66.26	7.31	0.981
Indoleamines/Catecholamines		1.74	0.11	1.85	0.26	0.838
<b>Hippocampus</b>		NA	134.21	5.84	126.75	8.27
	DA	28.86	4.19	26.18	3.29	0.619
	Total Catecholamines	157.71	6.67	151.68	8.65	0.846
	5-HIAA	118.24	6.64	141.23	7.76	<b>0.031*</b>
	5-HT	257.78	11.78	295.35	14.68	<b>0.054</b>
	Total Indoleamines	376.02	16.35	436.58	19.67	<b>0.024*</b>
	5- HIAA/5-HT	0.46	0.02	0.49	0.02	0.519
	NA/DA	6.47	1.25	6.63	1.39	0.979
	Indoleamines/Catecholamines	2.39	0.14	3.00	0.18	<b>0.012*</b>
	<b>Hypothalamus</b>	NA	2116.99	135.36	1913.08	101.48
L-DOPA		308.07	25.13	311.92	29.23	0.921
DOPAC		65.41	14.49	55.89	8.00	0.539
DA		293.10	23.93	310.12	19.58	0.583
HVA		240.94	20.37	338.56	32.17	<b>0.017*</b>
Total Catecholamines		2977.17	177.41	2854.74	122.88	0.567
5-HIAA		352.19	27.54	390.07	35.51	0.426
5-HT		967.30	71.29	1018.00	75.43	0.636
Total Indoleamines		1319.50	92.95	1408.07	105.42	0.545

5- HIAA/5-HT	0.37	0.02	0.39	0.02	0.729
DOPAC/DA	0.21	0.06	0.18	0.02	0.781
HVA/DA	0.86	0.09	1.16	0.14	0.108
NA/DA	7.47	0.44	6.48	0.46	0.343
(DOPAC+HVA)/DA	1.02	0.14	1.41	0.16	0.132
DOPAC+HVA	271.25	28.51	386.47	37.98	<b>0.020*</b>
Indoleamines/Catecholamines	0.45	0.02	0.50	0.04	0.220

Table 4. Neurotransmitter concentration (ng/g tissue) in amygdala, PFC, hippocampus and hypothalamus in pigs and the influence of the TI test in the response to control or stressful handling at the slaughterhouse. \*  $P < 0.05$

Variables	Control handling					Stressful handling					
	Non-fearful		Fearful		Effect	Non-fearful		Fearful		Effect	
	Mean	SE	Mean	SE		Mean	SE	Mean	SE		
Amygdala	NA	151.73	13.41	141.85	12.65	1.000	166.48	24.28	149.63	20.54	1.000
	DOPAC	45.62	10.00	49.15	6.43	1.000	37.47	7.18	40.66	8.71	1.000
	DA	407.38	59.12	399.67	30.76	1.000	278.69	46.24	352.17	50.24	0.583
	HVA	280.28	32.11	357.60	35.13	0.231	229.50	40.92	250.36	24.83	1.000
	Total Catecholamines	884.99	104.02	948.27	71.88	1.000	793.44	70.70	792.81	67.97	1.000
	5-HIAA	311.48	29.19	273.86	22.02	0.615	241.95	29.51	254.81	20.97	1.000
	5-HT	1016.53	73.68	1001.79	91.51	1.000	762.88	100.06	884.68	101.56	0.728
	Total Indoleamines	1328.02	98.50	1275.65	111.46	1.000	1004.83	125.00	1139.49	116.39	0.829
	5- HIAA/5-HT	0.31	0.02	0.28	0.01	0.603	0.33	0.02	0.31	0.03	0.837
	DOPAC/DA	0.10	0.01	0.12	0.01	0.666	0.11	0.01	0.11	0.02	1.000
	HVA/DA	0.75	0.08	0.91	0.09	1.000	1.18	0.35	0.81	0.12	0.904
	NA/DA	0.41	0.04	0.36	0.03	1.000	0.55	0.13	0.44	0.11	1.000
	(DOPAC+HVA)/DA	0.85	0.07	1.03	0.09	0.265	0.95	0.05	0.92	0.11	1.000
	DOPAC+HVA	325.89	39.97	406.75	39.03	0.260	290.98	40.62	291.01	29.00	1.000
	Indoleamines/Catecholamines	1.59	0.12	1.36	0.09	0.265	1.39	0.06	1.45	0.13	1.000
Prefrontal Cortex	NA	131.26	8.89	146.10	7.18	0.346	150.22	5.17	151.38	9.26	1.000
	DOPAC	8.46	1.85	6.86	1.74	0.737	7.15	1.91	6.59	1.65	1.000
	DA	16.17	2.69	23.03	2.85	0.119	20.46	2.71	19.80	1.52	1.000
	HVA	58.54	9.99	64.51	12.60	1.000	58.17	10.83	64.20	5.62	0.873
	Total Catecholamines	213.36	22.07	241.80	18.42	0.691	225.83	32.16	256.86	11.94	0.789
	5-HIAA	117.41	8.88	99.31	8.55	0.427	104.91	12.03	124.24	11.24	0.394
	5-HT	258.26	12.71	252.56	27.08	1.000	228.96	26.73	271.10	15.52	0.448
	Total Indoleamines	375.67	19.43	351.87	34.44	1.000	326.83	31.21	413.17	21.05	0.392
	5- HIAA/5-HT	0.46	0.03	0.41	0.03	0.589	0.48	0.06	0.46	0.02	1.000
	DOPAC/DA	0.46	0.06	0.28	0.06	0.167	0.39	0.10	0.35	0.09	0.945
	HVA/DA	3.82	0.39	2.84	0.47	0.292	3.45	0.64	3.34	0.44	1.000
	NA/DA	9.55	1.55	7.24	1.07	0.254	8.89	1.40	8.04	0.87	1.000
	(DOPAC+HVA)/DA	4.22	0.34	3.25	0.56	0.363	4.17	0.74	3.76	0.52	1.000

	DOPAC+HVA	65.94	11.74	70.61	14.16	1.000	63.54	12.59	69.38	7.26	0.968
	Indoleamines/Catecholamines	1.87	0.19	1.61	0.12	1.000	1.96	0.48	1.72	0.13	1.000
Hippocampus	NA	140.31	7.64	128.79	8.71	0.874	123.84	11.01	130.03	13.16	1.000
	DA	33.01	6.68	24.72	5.09	0.566	23.31	4.61	30.00	4.57	0.777
	Total Catecholamines	170.51	9.61	144.92	6.80	0.128	136.35	7.05	169.56	14.17	<b>0.047*</b>
	5-HIAA	119.86	10.72	116.81	8.75	1.000	134.88	8.42	148.38	13.70	0.733
	5-HT	253.05	21.67	261.98	12.42	1.000	265.13	13.90	329.35	22.04	<b>0.030*</b>
	Total Indoleamines	372.91	30.80	378.79	16.41	1.000	400.01	20.11	477.73	30.03	<b>0.063</b>
	5- HIAA/5-HT	0.48	0.03	0.45	0.04	1.000	0.51	0.03	0.46	0.04	0.537
	NA/DA	5.76	1.44	7.18	2.11	1.000	7.09	2.13	6.09	1.91	1.000
	Indoleamines/Catecholamines	2.15	0.19	2.62	0.16	0.290	3.09	0.27	2.88	0.26	1.000
Hypothalamus	NA	2011.75	177.09	2264.34	213.90	0.613	1724.17	128.95	2054.75	133.69	0.303
	L-DOPA	295.22	37.60	326.05	32.57	1.000	238.27	29.48	354.01	34.31	<b>0.090</b>
	DOPAC	58.26	16.61	74.94	28.97	1.000	57.50	13.78	54.27	9.52	1.000
	DA	293.43	41.27	292.63	14.28	1.000	286.96	35.20	327.50	21.95	0.713
	HVA	224.42	20.66	269.85	43.28	0.704	397.28	58.88	294.52	29.32	0.202
	Total Catecholamines	2858.10	254.02	3143.87	245.60	0.729	2624.76	72.66	3027.23	190.60	0.343
	5-HIAA	327.84	30.67	386.30	50.30	0.859	383.88	47.08	395.49	55.28	1.000
	5-HT	906.42	101.21	1052.53	94.46	0.714	900.42	114.09	1120.88	91.29	0.245
	Total Indoleamines	1234.26	128.72	1438.83	126.98	0.710	1284.30	155.95	1516.37	140.86	0.476
	5- HIAA/5-HT	0.38	0.03	0.37	0.04	1.000	0.43	0.02	0.35	0.03	0.120
	DOPAC/DA	0.16	0.03	0.28	0.13	0.842	0.19	0.03	0.16	0.03	1.000
	HVA/DA	0.85	0.13	0.88	0.13	1.000	1.45	0.25	0.94	0.13	<b>0.096</b>
	NA/DA	7.26	0.63	7.76	0.65	1.000	6.59	0.96	6.41	0.45	1.000
	(DOPAC+HVA)/DA	0.95	0.16	1.18	0.32	0.959	1.64	0.26	1.17	0.17	0.261
	DOPAC+HVA	257.71	31.60	294.94	60.51	1.000	454.78	67.95	335.23	36.59	0.270
	Indoleamines/Catecholamines	0.44	0.04	0.46	0.03	1.000	0.50	0.07	0.51	0.04	1.000

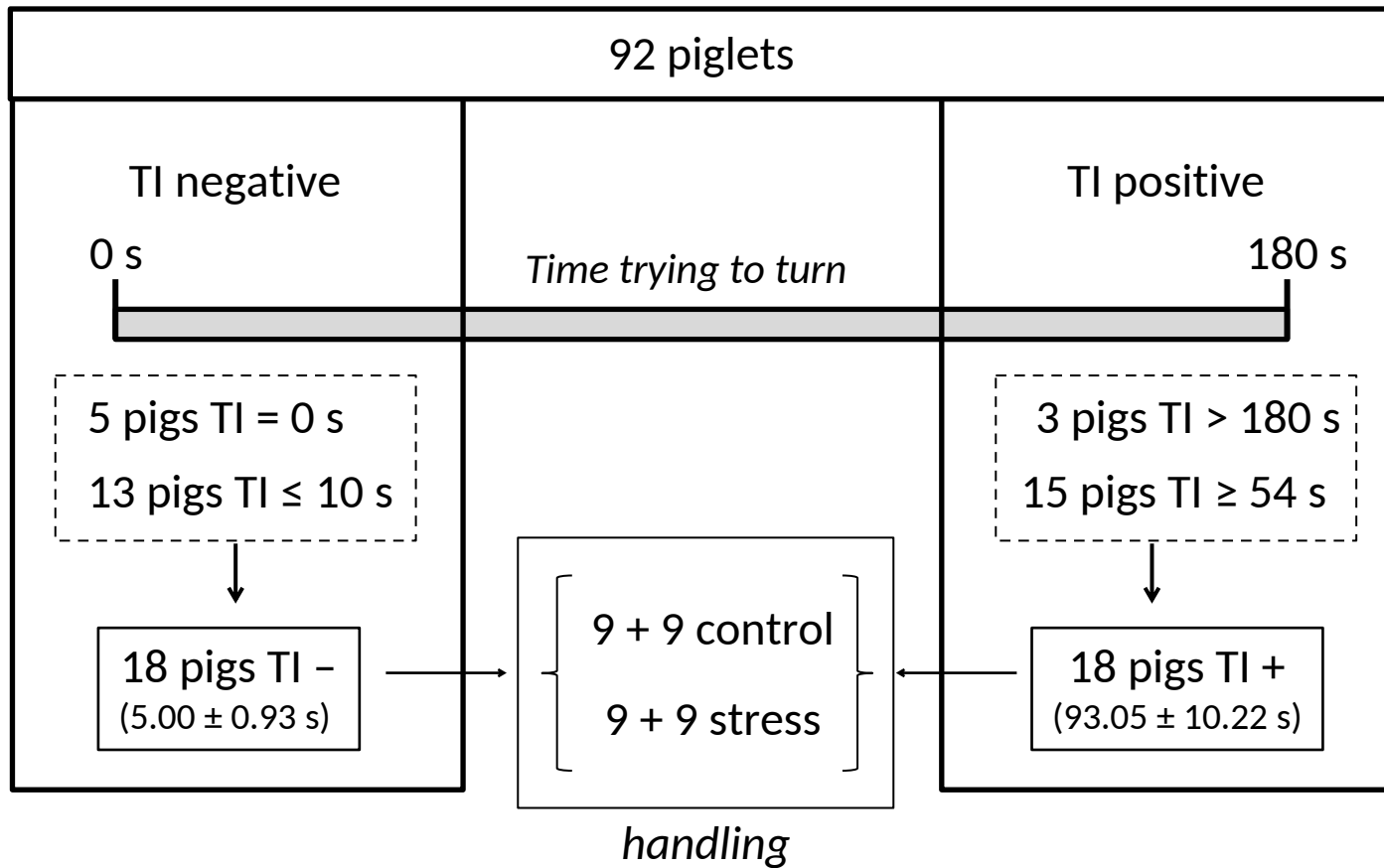


Figure 1

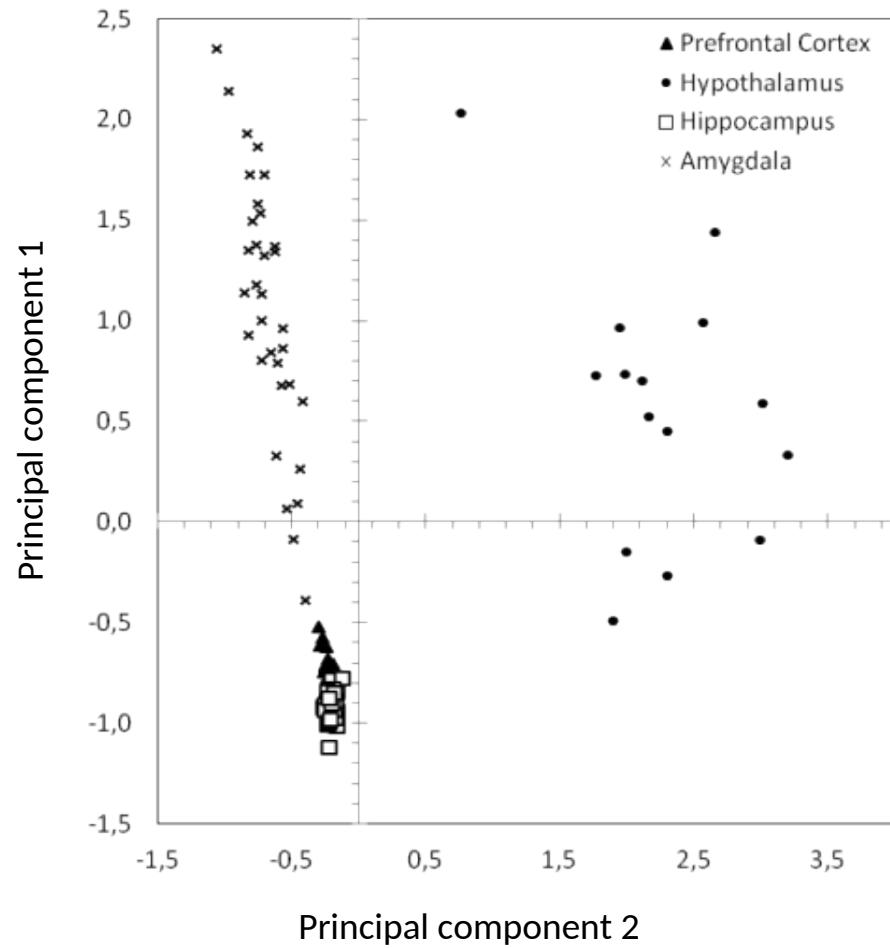


Figure 2

## **Highlights**

- We measured brain neurotransmitters in pigs classified as fearful-nonfearful under stressful handling.
- Stressful handling alters the 5-HT system in the hippocampus and the amygdala.
- There was no difference between fearful and non-fearful pigs under non-stressful handling.
- The 5-HT pathway is activated in the hippocampus under stressful handling only in fearful pigs.