Identification de structures cérébrales susceptibles d'être impliquées dans l'expression des comportements de peur chez les oiseaux, par une étude d'activation neuronale

Article en préparation

CHAPITRE 2 :
Identification de structures cérébrales susceptibles d'être impliquées dans l'expression des comportements de peur chez les oiseaux, par une étude d'activation neuronale.

Après s'être intéressé aux détails de l'implication de la seule structure connue pour être impliquée dans le contrôle des comportements de peur chez l'oiseau, l'arcopallium / PoA, nous avons cherché, dans ce deuxième chapitre, d'autres structures susceptibles de participer aux réseaux neuronaux contrôlant l'expression de ces comportements. En effet, étant donné la variété et la complexité des comportements de peur chez l'oiseau, il est probable que ces comportements soient dirigés par plusieurs circuits neuronaux mettant en jeu de nombreuses structures cérébrales**. Nous avons entrepris une approche exploratoire permettant de repérer des populations neuronales activées lors de l'expression des comportements de peur, considérant qu'une telle approche serait susceptible d'indiquer des régions pouvant participer au contrôle des réactions de peur. Pour cela, nous avons quantifié l'activation neuronale dans des structures candidates, en situation témoin et en réponse à une situation effrayante**.

Pour faciliter la mise en évidence de populations neuronales spécifiquement activées lors de l'expression des comportements de peur, nous avons utilisé des cailles des lignées STI et LTI, génétiquement sélectionnées sur leur propension à exprimer des comportements de peur. En effet, placées dans une même situation de peur, les cailles STI et LTI sont susceptibles de montrer une activation neuronale différente dans des régions jouant un rôle spécifique dans l'expression des comportements de peur. Il a par exemple été observé que deux lignées de rats aux comportements plus ou moins anxieux montrent une différence d'activation neuronale après exposition à un test d'open field dans des régions impliquées dans le contrôle des réactions d'anxiété, telle que le noyau paraventriculaire de l'hypothalamus (Salomé *et al*., 2004). Ainsi, à l'instar des modèles utilisés chez les rongeurs, la comparaison de l'activité cérébrale des cailles LTI et STI devrait faciliter la mise en évidence de structures spécifiquement activées lors de l'expression des comportements de peur.

Une étude d'activation neuronale nécessite au préalable la validation d'une situation de peur adaptée. En effet, il a été montré qu'un simple changement d'environnement induisait une forte activation neuronale dans des régions du cerveau non

spécifiques de la peur (Richard *et al.,* 2003). Or, la plupart des tests comportementaux utilisés pour induire des réactions de peur chez les oiseaux (par exemple, les tests d'immobilité tonique, d'open field …) comprennent un changement d'environnement. Nous avons donc choisi pour cette étude, un test qui évitait un changement d'environnement pour l'animal mais qui impliquait tout de même une stimulation forte et durable. Il s'agit de l'introduction soudaine d'un objet nouveau dans la cage d'élevage. La nouveauté est classiquement utilisée pour induire des réactions de peur chez l'animal (Bronson, 1968 ; Boissy, 1995 ; Jones, 1996). Une situation de test de présentation d'un objet nouveau dans la cage d'élevage a été mise au point au laboratoire. Il a été montré que cette situation induisait chez les cailles STI et LTI, des réponses physiologiques (augmentation de la corticostéronémie) et comportementales (évitement de l'objet et tentatives de fuite) caractéristiques d'un état de peur (Richard *et al.,* 2007b).

Pour étudier l'activation neuronale, nous avons utilisé un marqueur classique d'activation neuronale, la protéine FOS, produit d'expression du gène à expression précoce *c-fos*. Cette méthode a été largement utilisée pour visualiser l'activation neuronale dans diverses situations de peur chez les mammifères (Silveira *et al.,* 1993 ; Dielenberg *et al*., 2001 ; Salomé *et al*., 2004). Chez l'oiseau, cette méthode est aussi largement utilisée et a par exemple permis de cibler des structures cérébrales potentiellement impliquées dans l'expression des comportements sexuels chez la caille japonaise (Meddle *et al.,* 1999 ; Suge et McCabe, 2004 ; Heimovics et Riters, 2006).

Le but de cette étude fut donc d'étudier l'activation neuronale chez des cailles STI et LTI après exposition à un objet nouveau dans la cage d'élevage, en utilisant un marquage immunohistochimique de la protéine FOS. Le choix de structures candidates, au sein desquelles l'activation neuronale serait étudiée, a été établi sur la base des connaissances neuroanatomiques disponibles chez les oiseaux et des comparaisons possibles avec des structures connues chez les mammifères pour jouer un rôle dans l'expression des comportements de peur. Nous avons ciblé notre étude sur la mesure de l'activation neuronale dans l'arcopallium / PoA, le BST et le PVN et justifié ces choix **dans l'article présenté dans les pages suivantes. Au préalable, nous avons souhaité présenter les mises au point méthodologiques qui ont été nécessaires à la réussite de cette expérience.**

Chapitre 2, Etude préliminaire : Méthode de conservation des cerveaux et des coupes en vue d'améliorer le marquage immunohistochimique de la protéine FOS.

L'expérience visant à étudier l'activation neuronale à l'aide du marquage immunohistochimique de la protéine FOS a nécessité, au préalable, de traiter les cerveaux. Une fois le prélèvement des cerveaux réalisé, ils étaient tous immergés dans du paraformaldéhyde pendant 3 h puis placés dans une solution de sucrose 30 % à + 4°C, en attendant l'étape de coupe au cryostat. Les coupes de cerveaux étaient ensuite placées dans une solution d'antigel à -20°C, en attendant l'étape du marquage immunohistochimique. Compte tenu de ces différents traitements, nous n'avons pas été en mesure de traiter simultanément l'ensemble des cerveaux. Ainsi, les cerveaux ont été traités avec des délais différents entre le prélèvement et la coupe ou entre la coupe et le marquage. Or, lors des premiers essais de marquage immunohistochimique de la protéine FOS, nous avons observé que lorsque le marquage immunohistochimique était réalisé sur des cerveaux prélevés plus d'une semaine auparavant, le nombre de neurones marqués et l'intensité du marquage étaient très faibles.

Nous avons en premier lieu fait l'hypothèse que la méthode de conservation des coupes de cerveaux que nous utilisions (dans une solution d'antigel à -20°C) n'était pas adéquate. Pour tester cette hypothèse nous avons comparé cette méthode à une autre méthode de conservation utilisée au laboratoire (TBS-TA à +4°C) : 3 cerveaux de cailles ayant subi le même traitement ont été coupés le lendemain du prélèvement. La moitié des coupes de chaque cerveau ont été placés dans l'antigel à -20°C alors que les autres coupes étaient stockées dans le TBS-TA à + 4°C. Nous avons ensuite comparé la qualité du marquage immunohistochimique obtenu après conservation des coupes pendant 1 jour, 10 jours ou 30 jours. Les résultats ont montré qu'il n'y avait pas de diminution du marquage au cours du temps : nous n'avons pas observé de différence entre les coupes marquées 1 jour après la coupe et les coupes marquées 10 ou 30 jours après la coupe, quelle que soit la méthode de conservation utilisée. La méthode conservation des coupes dans l'antigel (à -20°C) que nous avions utilisée pendant les premiers essais, n'était donc pas à l'origine de la diminution de marquage observée au bout d'un certains temps.

Nous avons alors fait l'hypothèse que la conservation des cerveaux à +4 °C dans le sucrose avant la coupe au cryostat pouvait être à l'origine du problème. Pour tester cette hypothèse, nous avons investi en parallèle deux nouvelles méthodes de conservation des

cerveaux utilisées par des collègues, sur 6 cerveaux de cailles ayant subi le même traitement : 3 cerveaux placés à -20°C dans une solution cryoprotectrice pendant que 3 autres cerveaux ont été placés à -80°C sans cryoprotection. Nous avons ensuite testé différents délais entre le prélèvement et la coupe : 1 jour, 10 jours et 30 jours. Tous les cerveaux ont été marqués tout de suite après la coupe. Les résultats ont montré qu'il n'y avait pas de différences de marquage entre les cerveaux coupés 1 jour après le prélèvement et 10 ou 30 jours après, quelle que soit la méthode de conservation. La conservation des cerveaux à +4°C était donc responsable de la diminution du marquage observée après une semaine d'expérimentation, lors de notre expérience préliminaire.

Pour l'étude présentée dans ce chapitre, nous avons donc choisi de placer les cerveaux à -80°C sans cryoprotection, juste après le prélèvement (et passage dans le parformaldéhyde et sucrose) puis de conserver les coupes dans l'antigel à -20°C.

Fearfulness influences Fos expression in the paraventricular hypothalamic nucleus, bed nucleus of the stria terminalis and arcopallium / posterior pallial amygdala in Japanese quail.

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Abstract

This study investigated whether divergent genetic selection on a behavioural fear trait (tonic immobility duration) was related to change in neural activation in three brains areas potentially involved in the control of fear in birds. Fos expression was assessed in the arcopallium / posterior pallial amygdala (PoA) complex, the bed nucleus of the stria terminalis (BST) and the paraventricular nucleus of the hypothalamus (PVN) of quail selected for either short (STI) or long (LTI) tonic immobility duration, in two situations: sudden presentation of a novel object in their home cage vs. a control situation. Presentation of a novel object induced typical fear behaviour in STI and LTI quail and an increase in neural activation in the PVN of quail of the LTI line. In the control situation, neural activation was significantly higher in STI than in LTI quail in the anterior arcopallium and in the caudal part of the nucleus taeniae of the amygdala. Moreover, in the lateral part of the BST neural activation was significantly higher in LTI than in STI quail. These differences in neural activation between STI and LTI quail might underlie their differences in fear behaviour. In view of the known connections of these three structures in the avian brain, hypotheses linked to their potential involvement in fear behaviour in birds are discussed, taking into account data available in mammals.

Keywords:
Brain; Fear; Birds; Emotions; limbic system.

A major challenge of behavioral neuroscience is to understand the neural mechanisms of emotions in vertebrates. Most investigations in this field have involved mammals, in particular in the study of fear, an adaptive emotional response that is induced by the perception of a danger (Boissy, 1995; Jones, 1996). However, knowledge of the neural mechanisms of fear in birds is important for a better understanding of the phylogenic continuity of emotions. To facilitate the understanding of the neural mechanisms underlying fear, the comparison of animals with contrasting fearfulness, defined as the individual predisposition to react in threatening situations (Boissy, 1995), has proved to be useful in mammals (Landgraf & Wigger, 2002; Salomé *et al*., 2004; Zhang *et al*., 2004). In birds, two lines of quail divergently selected (Mills et Faure, 1991) for long (LTI) or short (STI) duration of an innate anti-predatory behaviour, tonic immobility (Gallup *et al*., 1971; Gallup, 1979), display different behavioural responses in a number of classical fear tests: compared to STI quail, LTI quail freeze more, vocalize and move less in an open field, emerge later from a "hole-in-the-wall" box (Jones *et al*., 1991), and struggle less during restraint in a "crush cage" (Jones *et al*., 1994). Moreover, genetic selection on tonic immobility was associated to changes in the activity of the autonomic nervous system in basal and fear-inducing situations (Valance *et al.,* 2007), responses of the hypothalamic-pituitary-adrenal (HPA) axis (Hazard *et al.,* 2005; 2007, 2008) and benzodiazepine and opiate receptors binding in the forebrain (Hogg *et al.,* 1994; 1996). Thus, STI and LTI quail exhibit differences at physiological levels, associated with the behavioural differences in fearfulness. To help in elucidating the neural mechanisms regulating fear behaviour in threatening situations, we assessed the neural activation evoked by a fear-inducing stimulus in STI and LTI quail, in brains areas potentially involved in the control of fear.

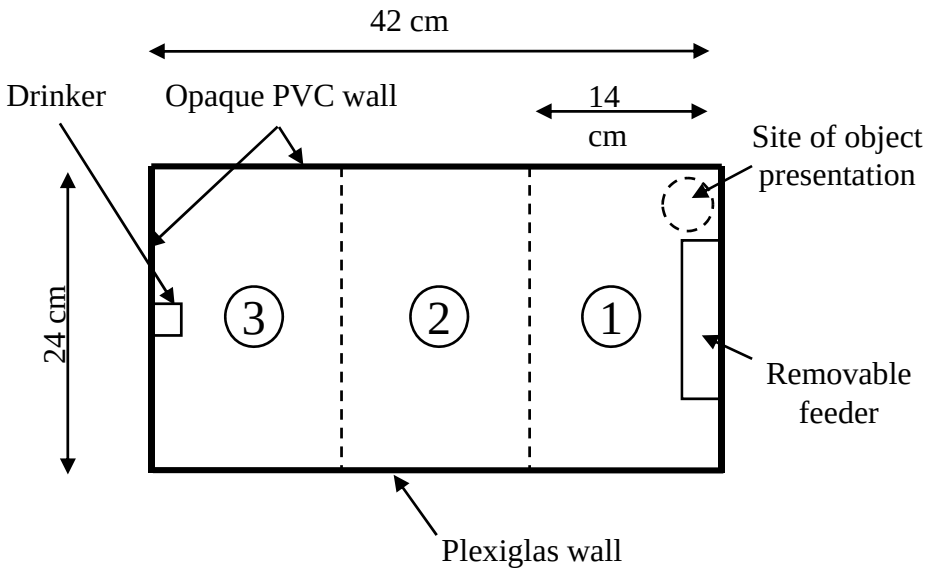
The literature available about fear in birds has pointed out the role of a brain region comprising the arcopallium and posterior pallial amygdala (arcopallium / PoA, originally called archistriatum; see Reiner *et al.,* 2004), this region being considered as partly homologous to the mammalian amygdala (Zeier & Karten, 1971; Davies *et al*. 1997a; Reiner *et al.* 2004; Atoji *et al.,* 2006). Indeed, studies using lesions or stimulations of the arcopallium / PoA complex have suggested a major role for this region in the expression of fear behaviour (Phillips & Youngren, 1971; Martin *et al.,* 1979; Phillips & Youngren, 1986; Lowndes & Davies, 1995). However, this brain region comprises many subdivisions (see Reiner *et al*., 2004) and the precise neuronal populations which may be specifically involved in the control of fear reactions have not been identified. In addition to the

arcopallium / PoA, the bed nucleus of the stria terminalis (BST) may be suspected to play a role in fear behaviour, but functional evidence is not available in birds. Indeed, the mammalian BST is known to participate in the neural control of fear (Walker *et al*., 2003) and similarities between the mammalian and the avian BST have been pointed out, based on their connections and neurochemical characteristics (Aste *et al.,* 1998; Richard *et al.,* 2004; Atoji *et al.*, 2006). Finally, the avian PVN is also suspected to play a role in fear responses, since it shares many connectional and neurochemical characteristics with the mammalian PVN and it is assumed to play a role similar to that of the mammalian PVN in the control of endocrine stress responses, notably the control of the activation of the HPA axis (Baylé *et al.,* 1975; Korf , 1984; Mikami, 1986). However, little evidence is available to confirm such a role. Therefore, the aim of the present study was to investigate fear-induced neural activation in the arcopallium / PoA, BST and PVN, three avian brain areas suspected to be involved in the control of fear behaviour.

Neural activation was assessed by quantifying the expression of the Fos protein, the product of the immediately-early gene c-fos, a widely used method for detecting neural activation in mammals as well as in birds. In birds, including quail, Fos immunohistochemistry has notably been used to investigate brain areas involved in the expression of several behavioural traits (Meddle, 1999; Suge & McCabe, 2004; Heimovics & Riters, 2006).

Fos expression was assessed in STI and LTI quail in two situations: sudden presentation of a novel object in their home cage vs. a control situation. Presentation of a novel object has been shown to induce typical physiological and behavioural fear reactions that are sustained over time, in STI as well as LTI quail (Richard et al., 2007; Saint-Dizier et al., 2007). It was expected that presenting a novel object might induce an increase in Fos expression in the three brain areas investigated, namely the arcopallium / PoA, BST and PVN and that Fos expression might differ between STI and LTI quail.

Figure 1: Diagram of a cage where the quail were housed after surgery and where the novel object test was performed (viewed from above). The diagram shows the site where the novel object was dropped and the imaginary boundaries delimiting the three zones used for behavior analysis



Materials and methods

Subjects

The experiment involved 40th generation Japanese quail (*Coturnix japonica)* selected for Long Tonic Immobility (LTI, n=24) and Short Tonic Immobility duration (STI, n=24), selected and maintained at the Unité Expérimentale Avicole, Nouzilly, France. The selection process has been continued since the foundation of the lines (Mills and Faure, 1991) and at the 40th generation, the mean duration of tonic immobility (± standard deviation), measured as in Mills & Faure (1991), was 228 (±80) s in the LTI line and 14 (±35) s in the STI line. In this experiment, male and female chicks of both lines were wing-banded on the day of hatching and transferred to a communal floor pen maintained at approximately 40°C by continuous illumination with commercial brooder lamps. Over the second and third weeks after hatching, the temperature was gradually reduced to 20°C and then stabilised. On the 21st day after hatching, the photoperiod was adjusted to a 16:8 h light:dark schedule. Standard food and water were freely available at all times, unless otherwise specified.

Throughout the experiment the birds were treated according to the European Communities Council Directive of November 24, 1986 (86/609/EEC). All procedures described here fully comply with French legislation on research involving animals.

Testing apparatus and procedure

At the 6th week of age, 48 adult males were transferred to a testing room maintained at 20°C and under a 16:8 h light: dark photoperiod. A single sex was used in this experiment made to reduce interindividual variability and males were chosen to avoid the changes in behaviour in females related to laying, which may occur unpredictably. The test apparatus and procedure have been described in detail previously (Richard *et al*. 2007). All quail were housed individually in PVC cages (see Figure 1 for measurements) with wood-shavings on the floor and an opaque PVC roof. The front wall of each cage was made of clear Plexiglas in order to be able to record the behaviour of quail with a camera during tests. This apparatus allowed introducing the object, a 21x4 cm multicoloured cylinder covered with 2-cm horizontal stripes of coloured tape, into the cage and pulling it out of the cage from a distance with minimal disturbance to the quail, the experimenter remaining out of sight of the bird during the procedure of object presentation and withdrawal.

Quail of both lines were randomly assigned to one of two groups: “Object” (LTI, n=12; STI, n=12) or control (LTI, n=12; STI, n=12). The test was performed eight or nine days

after the quail had been transferred into individual cages. On test days, the food trough of every cage was removed for 40 min before the test. To habituate the quail to this procedure, the food trough was removed from each cage for 2 h every day, during the week preceding testing. In the object situation, the food trough was replaced and the object was dropped into the cage as soon as the quail pecked at the food. If a quail failed to peck at the food, the object was dropped 60 s after the return of the food trough. The object was withdrawn after 5 min and the quail was left undisturbed for 1 min. The object presentation procedure was then repeated four more times at 1-min intervals, without waiting for the quail to peck in the food trough. In the control situation, the food trough was replaced and the behaviour of each quail was videoed without disturbance for 30 min from the moment they pecked at the food. If a quail failed to peck at the food, the video recording started 60 s after the return of the food trough.

Behaviour analysis

To analyse the locomotor behaviour of the quail, the cage was subdivided into 3 zones: zone 1 included the food trough and the site of object presentation; zone 2 was an intermediate zone and zone 3 was the area of the cage furthest away from the site of object presentation (Figure 1). The amounts of time spent in zones 1 and 3 were recorded. We also recorded the time spent pacing. Pacing was defined as repetitive movements fixed in form and orientation, including jumps and head movements.

The behaviour of "Object" quail was analysed during the first and fifth presentations of the object and the behaviour of control quail was analysed at corresponding time windows. All observations were made with The Observer 3.0 software (Noldus Information Technology, The Netherlands, 1993).

Brain collection and Fos immunohistochemistry

Two hours after the beginning of the behavioural observations, quail were deeply anesthetized with a lethal intraperitoneal injection of sodium pentobarbital (360 mg/kg; Sanofi Santé Animale, France). The thorax was opened and each bird was perfused through the left cardiac ventricule with 50 ml phosphate buffered saline (PBS; 0.1M, pH=7.4) followed by 250 ml 4% paraformaldehyde in PBS. Brains were then removed and immersed overnight in 4% paraformaldehyde in PBS at 4°C. They were subsequently cryoprotected by immersion in 30% sucrose in PBS for 24 hours at 4°C, frozen on dry ice and stored at -80°C until they were cut. Coronal sections (30 µm) were cut in a cryostat at -

20°C and stored in cryoprotectant (30% ethylene glycol and 20% glycerol in 5 mM low-salt PBS) at -20°C until used for immunohistochemistry. Every fourth section was used for Fos immunohistochemistry. Adjacent sections were collected onto glass slides and stained with cresyl violet to aid in histological localisation of the immunolabelling.

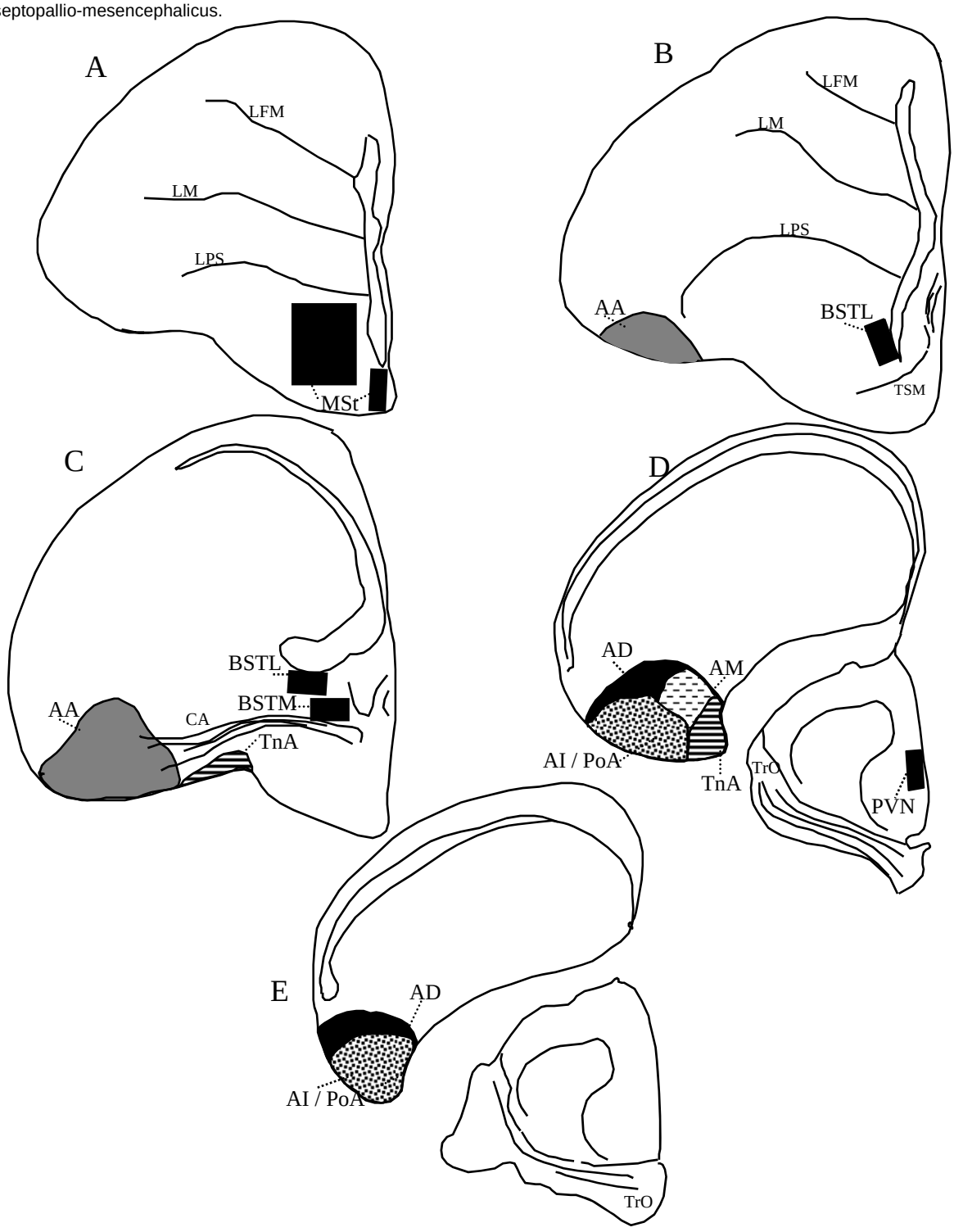
Immunohistochemistry was performed on free-floating sections. Unless otherwise specified, Tris-buffered saline (TBS; 0.5M, pH=7.6) was used to dilute reagents and to wash sections between steps. Sections were incubated for 72 hours at 4°C in primary antibody (sc 253 K-25, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted (1:10,000) in TBS containing 0.3% Triton X-100, 0.05% Thimerosal (Sigma, Saint Quentin Fallavier, France) and 1% bovine serum albumin (BSA, Sigma, Saint Quentin Fallavier, France). The primary polyclonal antibody was raised in rabbit against a 25 amino-acid sequence of the chicken Fos protein sequence. The sections were then incubated in 0.3% hydrogen peroxide at room temperature for 20 min in darkness to reduce endogenous peroxidase activity and were incubated with a biotinylated goat anti-rabbit secondary antibody (BA-1000, Vector Laboratory, Burlingame, CA, USA) diluted (1:2000) in TBS containing 0.3% Triton X-100, 0.05% Thimerosal and 2% goat serum for 24 hours at 4°C. After that, sections were incubated with the avidin-biotin complex (Vectastain ABC Kit, Vector Laboratory, Burlingame, CA, USA) diluted (1:2000) in TBS containing 0.3% Triton X-100 and 0.05% Thimerosal. Sections were then washed twice in TBS and twice in Sodium acetate buffer (NaAc; 0.1M; pH = 6.0) prior to visualization with a solution of 0.02 % diaminobenzidine (DAB, Sigma, Saint Quentin Fallavier, France), 0.01 % hydrogen-peroxide (H_2O_2), and 2.5 % Nickel-ammonium sulphate in NaAc. Sections were then mounted in 0.25% gelatine (Sigma, Saint Quentin Fallavier, France) in TBS onto glass slides, air-dried overnight at room temperature, dehydrated in 100% ethanol, defatted in xylene, mounted in DepeX (Sigma, Saint Quentin Fallavier, France) and coverslipped.

Quantification of Fos immunoreactivity

Quantification of Fos immunoreactivity was carried out using a light microscope with computerised image analysis system (Mercator, Explora Nova, La Rochelle, France). Fos immunoreactive cells were counted according to predetermined gray scale, form (only ovoid shapes were counted as positive nuclei) and size criteria (surface of immunopositive nuclei: 13 to 100 μm^2).

Figure 2: Schematic charts of frontal sections of quail brain illustrating the delineation of the fields used for quantification of Fos-immunoreactivity in the following regions: (A) MSt, used as a reference structure, at a level of the brain corresponding approximately to Plate A9.8 in Kuenzel & Masson's atlas of the chick brain (1988); (B) AA and BSTL, at a level of the brain corresponding approximately to Plate A9.0 in Kuenzel & Masson's atlas of the chick brain (1988); (C) AA, BSTL, BSTM and TnA, at a level of the brain corresponding approximately to Plate A8.2 in Kuenzel & Masson's atlas of the chick brain (1988) (D) different subdivisions within the arcopallium / PoA (AD; AM; AI /PoA; TnA) and PVN, at a level of the brain corresponding approximately to Plate A7.2 in Kuenzel & Masson's atlas of the chick brain (1988); (E) two subdivisions within the arcopallium / PoA (AI / PoA and AD), at a level of the brain corresponding approximately to Plate A6.2 in Kuenzel & Masson's atlas of the chick brain (1988).

AA: anterior arcopallium; AD: dorsal arcopallium; AI / PoA: intermediate arcopallium and posterior pallial amygdala; AM: medial arcopallium; BSTL: lateral part of the bed nucleus of the stria terminalis; BSTM: medial part of the bed nucleus of the stria terminalis; CA: commissura anterior; LFM: lamina frontalis suprema; LM: lamina mesopallialis; LPS: lamina pallio-subpallialis; MST: medial striatum; PVN: paraventricular hypothalamic nucleus; TnA: nucleus taeniae of the amygdala; TrO: tractus opticus; TSM: tractus septopallio-mesencephalicus.



Atlases of the domestic chick (Kuenzel & Mason, 1988) and Japanese quail (Baylé *et al.,* 1974) brains, together with the examination of cresyl violet staining, were used to aid in histological identification. The nomenclature used in the present paper is based on the revised nomenclature for the avian brain (Reiner *et al.,* 2004). Fos-immunoreactive cells were counted bilaterally in the medial striatum (used as a reference structure), bed nucleus of the stria terminalis, arcopallium / PoA complex and paraventricular hypothalamic nucleus, in sections that were 240 µm apart.

Medial striatum (MSt)

The MSt was chosen as a reference structure for this study because there is no specific hypothesis about its potential involvement in fear behaviour. In the MSt, Fos-ir cells were counted at 8 rostro-caudal levels, the most caudal level (MSt8) corresponding approximately to Plate A9.0 in Kuenzel & Masson's atlas of the chick brain (1988). The size of the quantification field was adapted to the size of the MSt (Figures 2A): area covered: 0.26 mm^2 for MSt 1 and MSt 2 and 0.9 mm^2 for MSt 3-MSt 8.

Bed nucleus of the stria terminalis (BST)

Two main subdivisions have been described in the avian BST (Aste *et al.,* 1998), these subdivisions being likely to subserve different functions. The lateral and medial parts of the BST were analysed independently.

In the lateral part of the BST (BSTL), Fos-ir cells were counted at 7 rostro-caudal levels, the most rostral level (BSTL1) corresponding approximately to Plate A9.0 in the chicken brain atlas (Kuenzel & Masson, 1988). The quantification field (0.1 mm^2 for BSTL 1-BSTL 3 and 0.125 mm^2 for BSTL 4-BSTL 7) was adjacent to the ventral horn of the lateral ventricle (Figures 2B & 2C).

In the medial part of the BST (BSTM), Fos-ir cells were counted at 3 rostro-caudal levels, the most rostral level taken into account (BSTM1) corresponding approximately to Plate 8.2 in Kuenzel & Masson's chicken brain atlas (1988). The quantification field (0.125 mm^2 for BSTM 1-BSTM 3) was adjacent to the dorsal edge of the anterior commissure (Figure 2C).

Arcopallium / posterior pallial amygdala (PoA) complex

In the arcopallium / PoA complex, Fos-ir cells were counted at 11 rostro-caudal levels, centred on the section corresponding approximately to Plate A8.2 in the chicken brain atlas (Kuenzel & Masson, 1988). The arcopallium / PoA complex was subdivided into five structures: the anterior arcopallium (AA; Figures 2B & 2C); the nucleus taeniae of

the amygdala (TnA; 2C & 2D); the medial arcopallium (AM; Figures 2D), the dorsal arcopallium (AD; Figures 2D & 2E) and the intermediate arcopallium and posterior pallial amygdala (AI / PoA; Figures 2D & 2E).

In the AA, Fos-ir cells were counted at six rostro-caudal levels, the most caudal level (AA6) corresponding approximately to Plate A8.2 in Kuenzel & Masson's atlas (1988) (AA6 to AA1). For the TnA and AM, 4 consecutive sections were analysed, whereas for the AD and AI/PoA, fives consecutive sections were analysed, the most rostral one corresponding to Plate A8.0 in Kuenzel & Masson's atlas of the chicken brain (1988) (TnA1, AM1, AD1, AI/PoA1 respectively).

P*araventricular hypothalamic nucleus (PVN)*

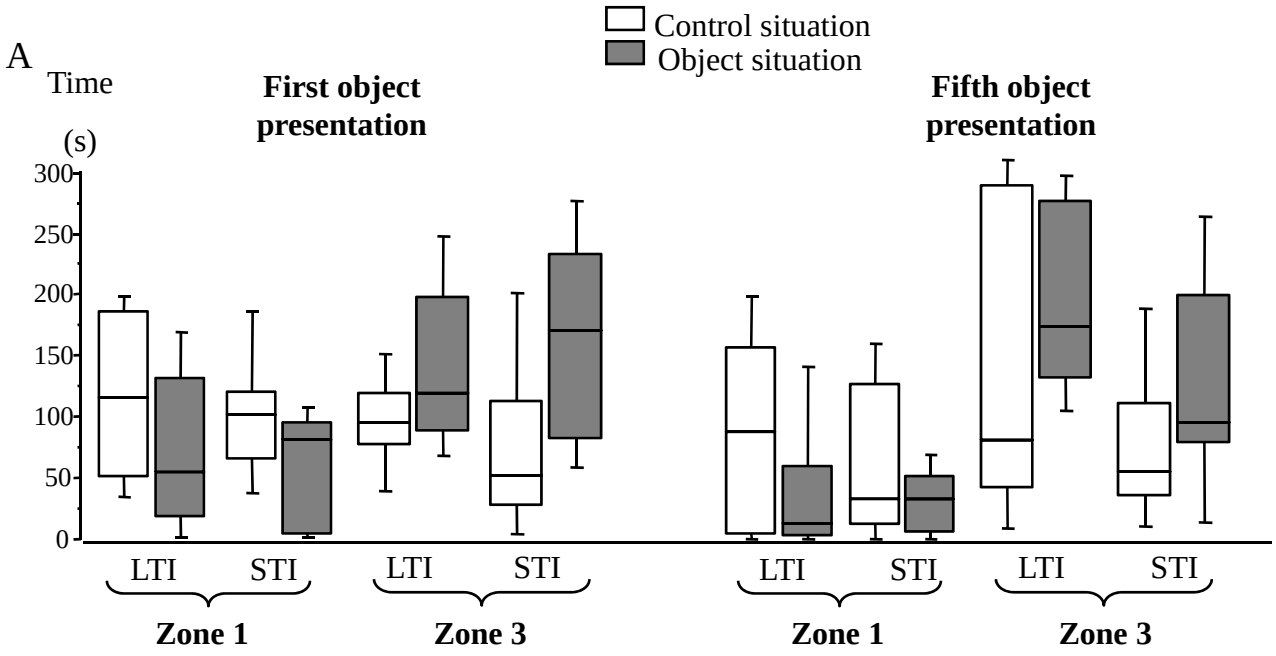
In the PVN, Fos-ir cells were counted at five rostro-caudal levels, the most rostral level corresponding approximately to Plate A7.8 in Kuenzel & Masson's atlas (1988). The quantification field was adjacent to the medial wall of the diencephalon and its size was adapted to the size of the PVN (0.1 mm^2 for PVN 1-PVN 5 and 0.15 mm^2 for PVN 2-PVN 4) (Figure 2D).

Within each structure under study, the density of Fos-ir cells in a given section was calculated as the mean of the densities for the right and left sides of the brain. In the regions of interest (BST, arcopallium / PoA and PVN) the results are expressed as a ratio of this local density relative to a reference density. For each quail, the reference density was the mean density of Fos-ir cells within the MSt of that quail. This normalisation of the data allowed to reduce interindividual variability. These normalised data were used for the statistical analysis.

Statistical analysis

The entire experimental procedure including behavioural observations, quantification of Fos-ir cells and statistical analysis was performed blind with respect to the line identity of the observed quail and to the experimental treatment of the birds. Because the behavioural data were not normally distributed, they were analysed with nonparametric statistical tests, using Statview 5.0 (SAS Institute Inc., USA, 1992-1998) and Excel 2000 (Microsoft Corporation) softwares. The effects of treatment (object *vs* control quail), line (LTI *vs*. STI quail) and interaction between treatment and line on the behavioural variables of interest (time spent in zone 1, time spent in zone 3, time spent eating and time spent pacing) were assessed using a 2x2 factorial analysis for unrelated

Figure 3: Box-plot diagrams illustrating (A) the time spent in two different zones of the cage and (B) the time spent pacing, in quail selected for long (LTI) or short (STI) tonic immobility duration during the first and the fifth presentations of a novel object (grey bars) and during a control situation (open bars). Zone 1 = area of the cage including the food trough and the site of object presentation. Zone 3 = area of the cage furthest away from the object. n = 12 for each bar.

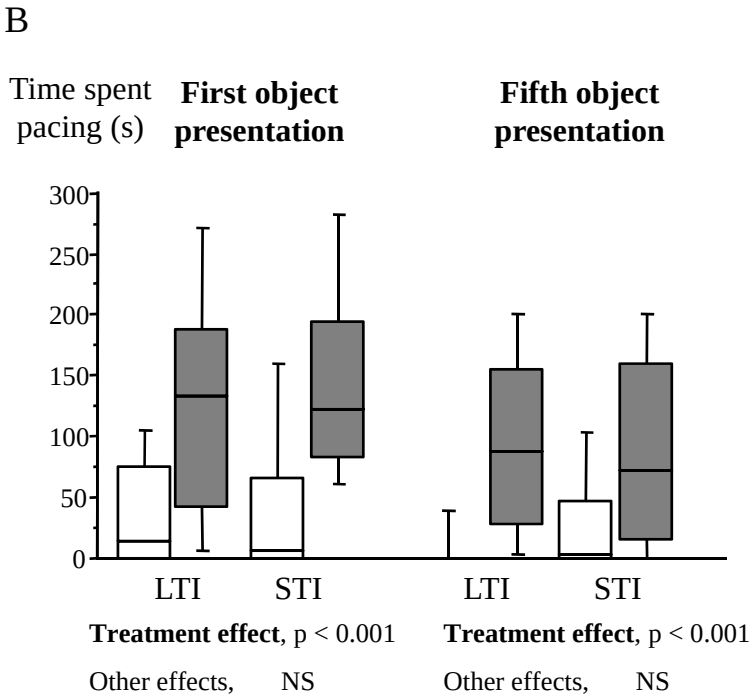


Treatment effect, $p < 0.05$ — Other effects, NS

Treatment effect, $p < 0.01$ — Other effects, NS

All effects, NS

Treatment effect, $p < 0.05$ — **Line effect,** $p < 0.05$ — Interaction, NS



Treatment effect, $p < 0.001$ — Other effects, NS

Treatment effect, $p < 0.001$ — Other effects, NS

datasets, with non-specific hypotheses (Meddis, 1984). Evolution of the variables between the first and fifth object presentations was analysed within each experimental group using Wilcoxon tests. Box-plot diagrams were used to illustrate the behavioural results. Values illustrated by a box-plot are the median, upper and lower quartiles, 10^{th} and 90^{th} percentiles.

A two-way ANOVA with treatment and line as factors was carried out on the mean density of Fos-ir cells in the MSt (used as a reference density). In the regions of interest, statistical analyses were performed on the normalised densities of Fos-ir cells, using a two-way ANOVA for repeated measures (different rostrocaudal levels within a region) with treatment and line as factors. When the interaction between treatment and line was significant, the analysis was followed by a one-way ANOVA for repeated measures within each line. When the interaction between rostrocaudal level and line was significant, a two way ANOVA with line and treatment as factors was performed for each rostrocaudal level. Throughout the text and figures, the normalised density of Fos-ir cells was expressed as the mean and standard error of the mean within each experimental group. In all analyses, $p<0.05$ was considered as significant.

Results

Behaviour (Figure 3)

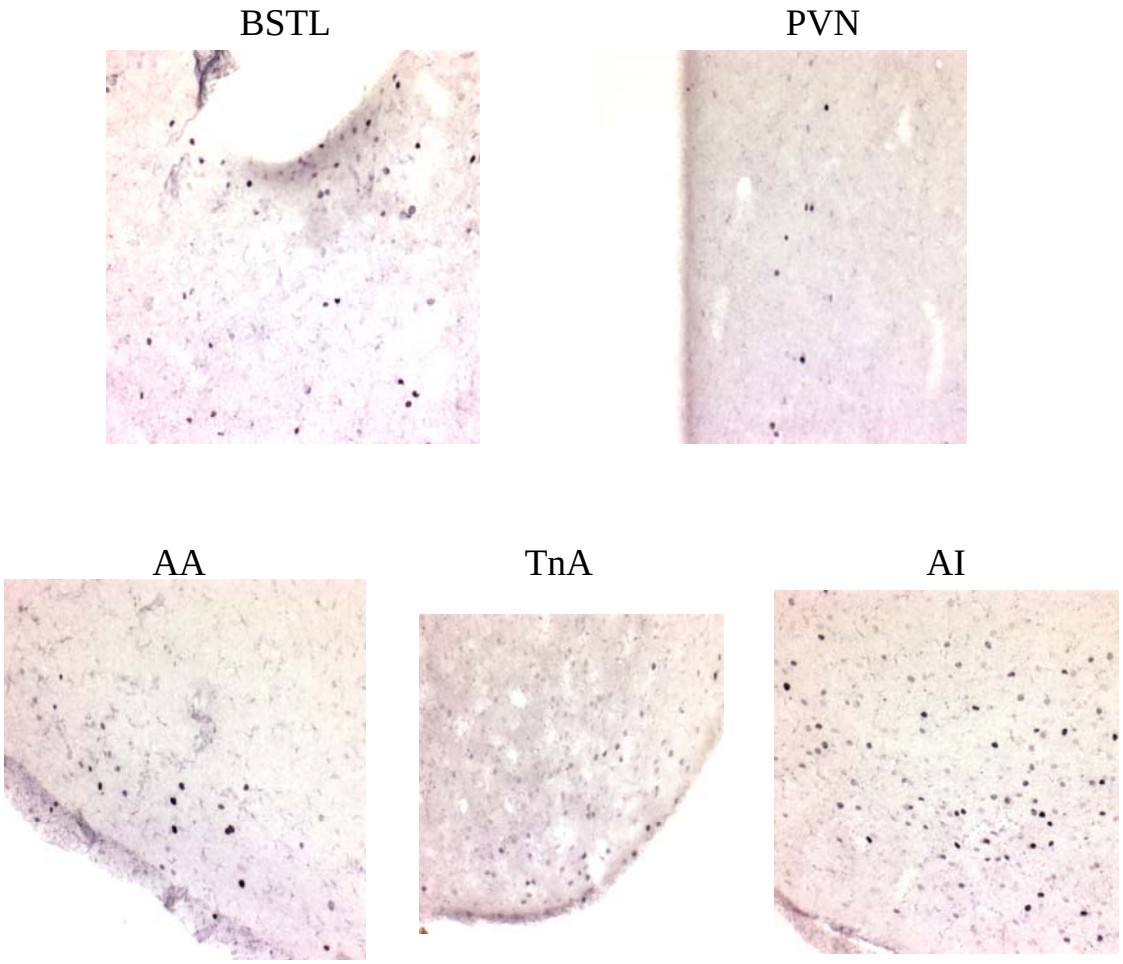
Behaviour during the first object presentation

There was an effect of treatment on the time spent in zone 1 ($H = 5.24$, $p < 0.05$), the time spent in zone 3 ($H = 9.19$, $p < 0.01$) and the time spent pacing ($H = 16.17$, $p < 0.001$). 'Object' quail spent significantly less time in zone 1, more time in zone 3 and more time displaying pacing than control quail. There was neither a significant effect of line ($H < 0.24$ for each variable), nor a significant effect of the interaction between line and treatment on any variable of interest ($H < 1.24$ for each variable).

Behaviour during the fifth object presentation

There was an effect of treatment on the time spent in zone 3 ($H = 4.55$, $p < 0.05$) and on the time spent pacing ($H = 14.32$, $p < 0.001$): 'Object' quail spent significantly more time in zone 3 and more time displaying pacing than control quail. There was no significant effect of treatment on the time spent in zone 1 ($H = 1.97$). There was a significant effect of line on the time spent in zone 3 ($H = 4.51$, $p < 0.05$): quail of the LTI line spent significantly more time in zone 3 than quail of the STI line. There was neither a

Figure 4: Photomicrographs illustrating the density of Fos-ir cells within the regions of interest. AA: anterior arcopallium; AI: intermediate arcopallium; BSTL: lateral part of the bed nucleus of the stria terminalis; PVN: paraventricular hypothalamic nucleus; TnA: nucleus taeniae of the amygdala



significant effect of line on any other variable (time spent in zone 1 and time spent pacing; $H < 0.73$ for each variable) nor a significant effect of the interaction between line and treatment on any variable of interest ($H < 1.46$ for each variable).

Behavioural changes over time

There was no significant change between the first and fifth period on any variable of interest ($z < 1.8$ for each comparison, Wilcoxon tests).

Fos immunoreactivity

Fos-ir cells, characterised by dense dark nuclear staining, were observed in all studied brains regions with varying densities, as illustrated in Figure 4.

Medial striatum (MSt)

There was no significant effect of treatment ($F = 1.14$), line ($F = 1.89$) or interaction between line and treatment ($F = 0.147$) on the density of Fos-ir cells in the MSt.

Bed nucleus of the stria terminalis (BST) (Figure 5)

In the BSTL (Figures 4 & 5A), there was a significant effect of line on the normalised density of Fos immunoreactivity ($F = 5.55$; $p < 0.05$), LTI quail showing a higher density of Fos-ir cells than STI quail. There was neither a significant effect of treatment ($F = 0.35$) nor an effect of interaction between line and treatment ($F = 0.51$) on the density of Fos-ir cells in the BSTL.

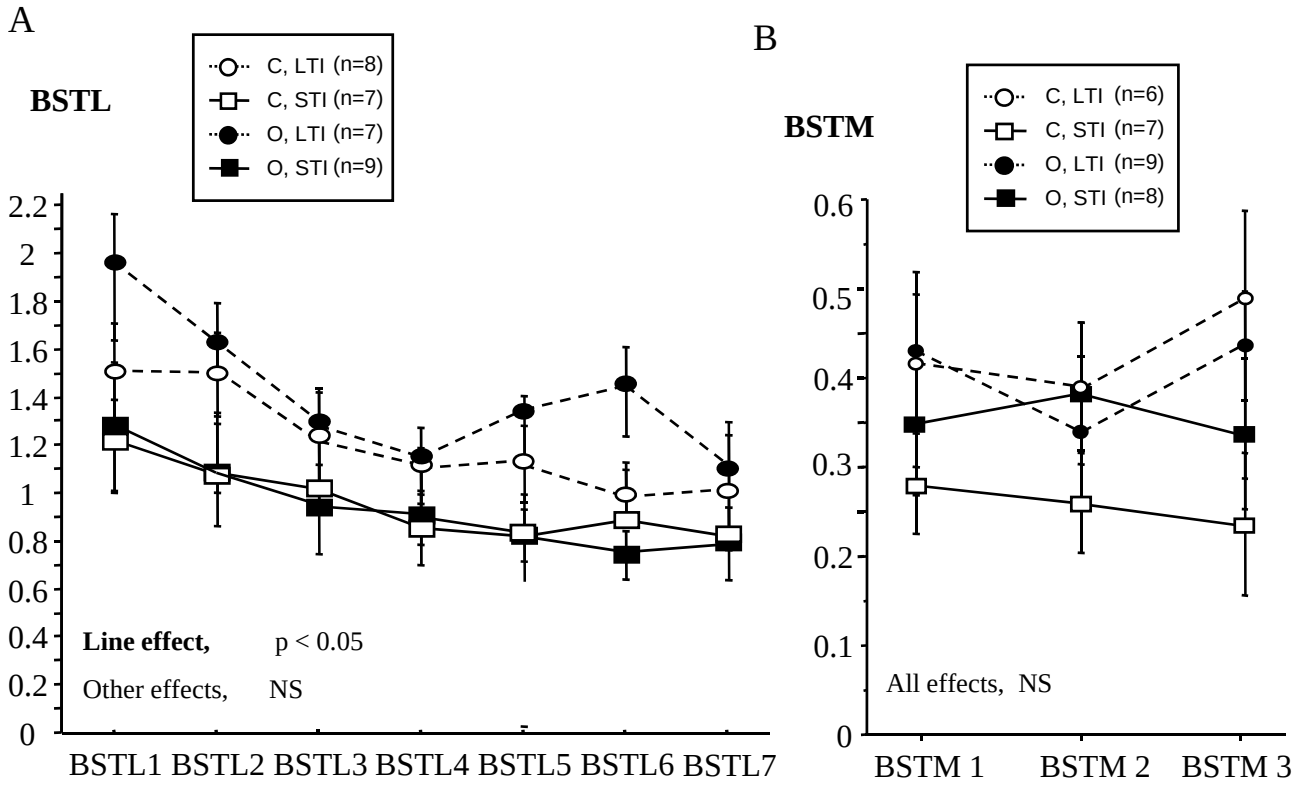
In the BSTM (Figure 5B), there was no significant effect of treatment ($F = 0.25$), line ($F=2.56$) or interaction between line and treatment ($F = 0.86$) on the normalised density of Fos-ir cells.

Arcopallium / posterior pallial amygdala complex (Figure 6)

There was a significant effect of line on the normalised density of Fos-ir cells in the entire arcopallium / PoA ($F = 6.24$; $p < 0.05$): LTI quail showed a lower normalised density of Fos-ir cells than STI quail. There was neither a significant effect of treatment ($F = 2.03$) nor an effect of interaction between line and treatment on the density of Fos-ir cells in the entire arcopallium / PoA ($F = 2.0$).

In the AA, there was a significant effect of line on the density of Fos-ir cells ($F = 5.13$; $p < 0.05$): LTI quail showed a lower normalised density of Fos-ir cells than STI quail. There was neither a significant effect of treatment ($F = 2.58$) nor an effect of

Figure 5: Normalised density of Fos-ir cells in the BSTL (A) and BSTM (B). Within each structure the results are shown for each level analysed, from the most rostral (BSTL1 & BSTM1) to the most caudal (BSTL7 & BSTM3).
BSTL: lateral part of the bed nucleus of the stria terminalis; BSTM: medial part of the bed nucleus of the stria terminalis



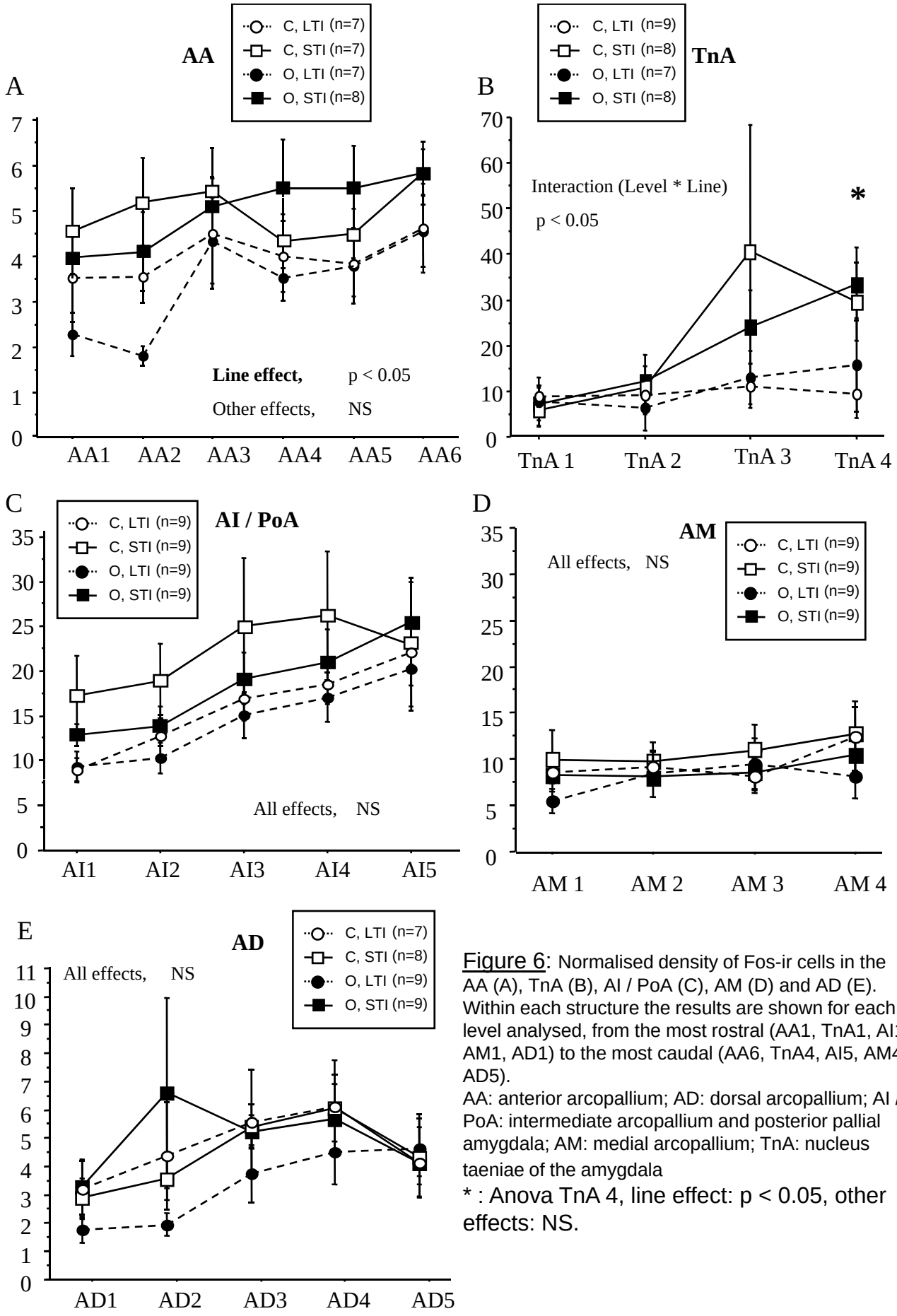
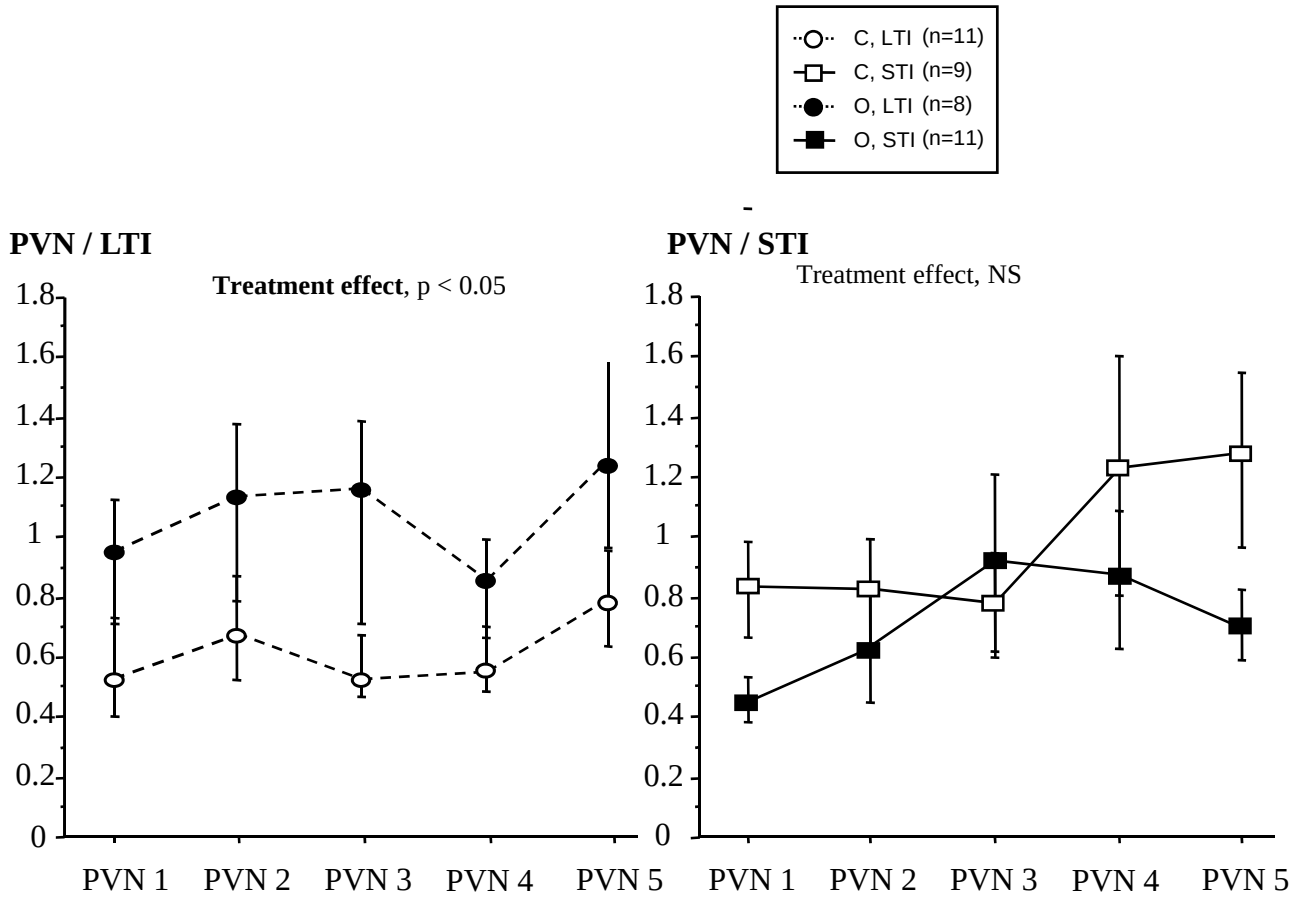


Figure 6: Normalised density of Fos-ir cells in the AA (A), TnA (B), AI / PoA (C), AM (D) and AD (E). Within each structure the results are shown for each level analysed, from the most rostral (AA1, TnA1, AI1, AM1, AD1) to the most caudal (AA6, TnA4, AI5, AM4, AD5).

AA: anterior arcopallium; AD: dorsal arcopallium; AI / PoA: intermediate arcopallium and posterior pallial amygdala; AM: medial arcopallium; TnA: nucleus taeniae of the amygdala

* : Anova TnA 4, line effect: $p < 0.05$, other effects: NS.

Figure 7: Normalised density of Fos-ir cells in the PVN (paraventricular hypothalamic nucleus) in quail of the LTI line (A) and quail of the STI line (B). The results are shown for each level analysed, from the most rostral (PVN1) to the most caudal (PVN5).



interaction between line and treatment on the normalised density of Fos-ir cells in the AA ($F = 0.06$) (Figure 6A).

Within the TnA, there was a significant effect of interaction between the rostrocaudal level and the line ($F = 3.58$; $p < 0.05$) on the normalised density of Fos-ir cells (Figure 6B). At the most caudal level taken into account within the TnA (TnA4), there was a significant effect of line on the normalised density of Fos-ir cells ($F = 5.14$; $p < 0.05$), LTI quail showing a lower normalised density of Fos-ir cells than STI quail. There was neither a significant effect of treatment ($F = 0.86$) nor an effect of interaction between line and treatment in TnA4 ($F = 0.17$). In TnA1, TnA2 and TnA3, there was no significant effect of treatment ($F < 0.33$), line ($F < 1.89$) or interaction between line and treatment ($F < 0.31$) on the normalised density of Fos-ir cells.

In the AM, AD and AI/PoA, there was no significant effect of treatment ($F < 0.91$), line ($F < 2.70$) or interaction between line and treatment ($F < 0.78$) on the normalised density of Fos-ir cells in the AM (Figure 6C, 6D & 6E).

Paraventricular hypothalamic nucleus (PVN)

There was a significant effect of interaction between line and treatment ($F = 5.73$; $p < 0.05$) on the normalised density of Fos-ir cells in the PVN. One-way ANOVAs revealed a significant effect of treatment in the LTI line ($F = 6.05$; $p < 0.05$) but not in the STI line ($F=1.19$): “Object” LTI quail showed a higher density of Fos-ir cells in the PVN than control LTI quail (Figure 7).

Discussion

Presentation of a novel object induced an increase in neural activation in the PVN of quail of the LTI line, which are characterised by a high level of fearfulness, but not of quail of the STI line. Moreover, differences in neural activation between the two lines were observed in the arcopallium / PoA and the BSTL, in basal conditions. These results will be discussed in terms of potential involvement of the structures under study in the control of fear behaviour.

Fos expression induced by presentation of a novel object

Presentation of a novel object into the home cage enhanced Fos expression in the PVN in quail of the LTI, but not in quail of the STI, line, suggesting fear-induced activation of the PVN in LTI quail. To our knowledge, a similar activation of the avian PVN has only been described once, in response to human handling (Goodson & Evans, 2004). In mammals, the PVN is activated in response to a variety of fear and anxiety-inducing stimuli (Dielenberg *et al.,* 2001; Silveira et al., 1993; 2001; Salomé *et al*., 2004; Singewald *et al*., 2003). It is involved in the control of the HPA axis and thus represents an important final pathway in the integration of endocrine stress responses (for review, see Herman *et al*., 2005). The results of the present study, together with the known homologies between the avian and mammalian PVNs (Baylé *et al*., 1975; Korf, 1984, Mikami, 1986), suggest that the avian PVN may play a similar role. Thus, cellular activation in the PVN in response to presentation of the novel object in LTI quail probably reflects an activation of the HPA axis. Such hypothesis is strengthened by the fact that in LTI quail, presentation of the novel object induces an increase in the levels of plasma corticosterone, the major glucocorticoid in birds (Richard *et al*., 2007). However, the increase in corticosterone concentrations after exposure to the novel object does not differ between STI and LTI quail (Richard *et al.,* 2007), whereas in the present study, the increase in Fos expression in the PVN was observed in quail of the LTI line, but not in the quail of the STI line. This apparent discrepancy might be explained by differences between STI and LTI quail in pituitary responsiveness to hypothalamic corticotropin-releasing factor (CRF). Indeed, pituitary responsiveness to CRF appears to be more pronounced in STI than in LTI quail (Hazard *et al.,* 2007). Thus, the similar levels of plasma corticosterone observed in STI and LTI quail in response to the presentation of the novel object, may be the consequence of a greater release of CRF by PVN neurons of LTI quail, in comparison to STI quail. The greater level of Fos expression observed in the PVN of LTI quail in the present study is

congruent with this hypothesis, but additional studies will be necessary to further characterise fear-activated neurons within the avian PVN and evaluate their involvement in endocrine stress responses.

In the present study, since the PVN was the only structure where an increase in Fos expression was detected after presentation of a novel object, it may be argued that the introduction of a novel object into a quail's home cage is not an appropriate fear test. But, in the present experiment, this test induced typical fear reactions in LTI as well as in STI quail (escape attempts, avoidance of the novel object), as previously shown by Richard *et al.* (2007). We conclude that introducing a novel object into the home cage is a good way to induce fear reactions in quail. Moreover, the novel object test is carried out in the home cage of the birds, preventing undesirable neural activation that would be induced by transferring the birds into a novel environment, such a transfer being a prerequisite for many fear tests in birds (Richard *et al.*, 2003). Nevertheless, Richard *et al.* (2007) note that presentation of a novel object induces a lower rise in plasma corticosterone levels than restraint in a crush cage, suggesting that quail do not perceive presentation of a novel object as the most frightening situation. Thus, it is possible that the presentation of a novel object was not strong enough to induce detectable Fos expression in a large population of neurons.

Differential Fos expression between quail of the STI and LTI lines in basal conditions:

The differences in neural activation observed in basal conditions between lines of quail differing in their levels of fearfulness, namely the STI and LTI lines, associated with connectional and neurochemical knowledge available in the avian brain, may point out to brain areas potentially involved in the control of fear behaviour. Indeed, these neural differences between the two lines of quail may underlie their differences at the behavioural level in fear-inducing situations.

In the arcopallium / PoA, Fos expression was significantly higher in STI than in LTI quail. Several functional studies have already demonstrated that the arcopallium / PoA complex is involved in the expression of fear reactions in birds (Phillips, 1964; Phillips & Youngren, 1971; 1986; Lowndes & Davies, 1995). It has been suggested that this brain region could be partially homologous with the mammalian amygdala (Zeier & Karten,

1971; Davies *et al*., 1997a), which is known to play a major role in fear behaviour in mammals (for review see LeDoux, 2000). Moreover, differences between STI and LTI quail have previously been reported in the arcopallium / PoA complex. Indeed, electrolytic lesions of this brain region induce a decrease in fear behaviour in quail of the LTI line, but not in quail of the STI line (Davies *et al*., 1997b). Recent studies have also shown that the volume of the arcopallium / PoA is greater in STI than in LTI quail (Richard *et al.*, 2005), and this result has been confirmed in the present study (data not shown). Therefore, both morphological and functional differences within the arcopallium / PoA complex, including the difference in neural activation observed in the present study, might underlie the differences in fear behaviour between these two lines.

Within the arcopallium / PoA, the difference in Fos expression between lines was most pronounced in the anterior arcopallium (AA). Such a result was surprising because, on the basis of the connections of the arcopallium / PoA, it has previously been suggested that the intermediate arcopallium (AI) and PoA might play a major role in limbic functions, whereas the AA might be more specifically involved in somatosensory and motor functions (Zeier & Karten, 1971; Davies *et al*., 1997a). The results of the present study suggest that the anterior arcopallium may play a role in the control of fear behaviour. In support of such a hypothesis, a specific involvement of the AA in the control of fear behaviour has recently been demonstrated, since lesions of the AA have been shown to induce a decrease in fear behaviour in LTI quail (Saint-Dizier *et al*., submitted). This involvement of the AA in fear behaviour is also supported by hodological data in the domestic chick, since Davies *et al*. (1997a) have reported projections from the ventral part of the AA to structures that are traditionally considered to be limbic in nature, such as the hippocampal formation, nucleus accumbens and BSTL. In contrast, Fos expression did not differ between STI and LTI quail in the AI / PoA, although this brain region is known to project to limbic structures such as the BSTL, the hippocampal formation, nucleus accumbens and hypothalamus (Zeier & Karten, 1971; Davies *et al*., 1997a; Atoji *et al*., 2006). However, we cannot exclude that, in the present experiment, activation of the AI / PoA differed between experimental groups, since neural activation may trigger other cellular pathways than those involving Fos (Hoffman, 2002). Thus, the present results support the involvement of the AA, but do not disprove the involvement the involvement of the AI / PoA, in fear behaviour.

Within the caudal part of the nucleus taeniae of the amygdala (TnA), Fos expression was significantly higher in STI than in LTI quail. The TnA has previously been

suggested to be limbic in nature, based on its neurochemical characteristics (Yamamoto *et al.,* 2005). More precisely, it has been suggested that the TnA may be the avian equivalent of at least certain parts of the mammalian amygdala, notably the medial and central nuclei of the amygdala (Cheng *et al.,* 1999; Absil *et al*., 2002; Yamamoto *et al.,* 2005). As these amygdala nuclei are involved in fear behaviour in mammals (Li *et al*., 2004; Takahashi *et al.*, 2007; LeDoux, 2000), it is possible that the avian TnA plays a similar function in birds. The TnA has predominantly been studied for its involvement in sexual behaviour (Absil *et al.,* 2002; Yamamoto *et al*., 2005), even though Cheng *et al*. (1999) have previously attributed the effect of lesions of the TnA in doves to a reduction in fearfulness. Since differences in sexual behaviour between LTI and STI quail have been reported (Burns et al., 1998), the differential neuronal activation between the two lines within the TnA observed in the present study may also be interpreted in terms of its potential involvement in sexual behaviour. Moreover, in the present study, the difference between STI and LTI quail was only observed in the caudal part of the TnA, which is congruent with anatomical and functional studies indicating that the avian TnA is not a homogeneous structure (Absil *et al*., 2002; Sun *et al*., 2005; Yamamoto *et al*., 2005). Additional studies will be necessary to identify precise subdivisions within the TnA and test their potential involvement in fear behaviour in birds.

In the BST, Fos expression was significantly higher in LTI than in STI quail in the lateral (BSTL), but not in the medial (BSTM) part. Neurochemical and connectional evidence suggests that the avian BSTL may be comparable to the BST of mammals (Reiner *et al.,* 2004; Richard *et al.,* 2004; Atoji *et al*., 2006), which is known to be involved in the control of unconditioned fear behaviour (Walker *et al.,* 2003, Sullivan *et al.,* 2004). There is little functional evidence about the role of the avian BSTL, even though an activation of this structure has previously been described after human handling (Goodson & Evans, 2004). Additional investigations will be necessary to demonstrate the precise role of this brain region in fear behaviour. In contrast, the avian BSTM has been mainly studied for its involvement in the control of sexual behaviour (Aste *et al*., 1998; Panzica *et al*., 2001), and there is no evidence to suggest that this brain region is involved in fear behaviour. Therefore, the possible involvement of the avian BST in fear behaviour is more likely to lie in its lateral, than in its medial, subdivision.

In summary, fear-induced neural activation in the PVN of LTI quail, likely reflected an activation of the HPA axis. Moreover, the description of a differential neural activation in different subdivisions of the arcopallium / PoA and BST between STI and LTI quail, known to differ in their fearfulness, brings new evidence for a functional parcellation of these structures and emphasizes the value of the STI / LTI model for the study of brain areas involved in the control of fear behaviour in birds. Additional investigations will be necessary to precise the specific roles of the BSTL, PVN and of the different subdivisions of the arcopallium / PoA in the neural control of fear reactions in birds.

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Figure Chapitre 2: **Résultats annexes**

Volume de l'arcopallium / PoA, de l'AP et de l'AA chez les cailles des lignées STI et LTI (droit + gauche, en mm^3)