Feather pecking and monoamines

A behavioral and neurobiological approach

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Verenpikken en monoamines

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(met een samenvatting in het Nederlands)

Proefschrift

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"Which way you ought to go depends on where you want to get to..."
— Lewis Carroll, *Alice in Wonderland*

TABLE OF CONTENTS

Chapter 1

General introduction

THE PROBLEM OF FEATHER PECKING IN LAYING HENS

Feather pecking (FP) remains one of the major welfare issues in laying hens. In particular the pecking at and pulling out of feathers, called severe feather pecking (SFP), inflicts damage to the plumage and causes denuded areas on the body of the recipient (Hughes and Duncan, 1972; Savory, 1995). The created bald spots will attract others to peck at the skin, and therefore SFP can easily progress into cannibalism causing mortality (Savory and Mann, 1997). SFP can be discriminated from gentle feather pecking (GFP) and aggressive pecking (Savory, 1995). GFP is a mild form of feather pecking without damage to the feathers or skin (Keeling and Jensen, 1995; Kjaer and Vestergaard, 1999). The above mentioned pecking behaviors clearly differ in the target to which they are directed. GFP and SFP are mostly directed at the back, tail and wing feathers (Riedstra et al, submitted, Savory, 1995), whereas aggressive pecking is almost exclusively directed at the top of the head or the comb. Aggressive pecking serves to establish or maintain the hierarchy and usually forces the recipient to react (Wennrich, 1975). It has been suggested that GFP is a form of explorative social pecking (Riedstra and Groothuis, 2002), although GFP can also be observed in multiple bouts with a stereotypic character (Kjaer and Vestergaard, 1999; McAdie and Keeling, 2002). SFP is, without any doubt, detrimental behavior and can be interpreted as a behavioral disorder (van Hierden et al., 2002; van Hierden et al., 2004; van Zeeland et al., 2009; Bordnick et al., 1994).

Beak trimming, i.e. the removal of the sharp tip of the beak, is an effective measure to reduce the damage inflicted by SFP. Nonetheless, this method may cause welfare problems in itself since the (removed) tip of the beak contains many nerve endings enabling the discrimination between pecked objects/food (Freire et al., 2011; Gentle, 1997). Behavioral studies indicate that beak-trimmed animals may chronically suffer from painful beak deformations and chronic neuroma formation (Gentle, 1986; Duncan et al., 1989). Considering this, the European legislation has regulated beak trimming and the Netherlands will prohibit beak trimming in 2018. Similar decisions are being prepared in Germany and the United Kingdom. Beak-trimming is already prohibited in Sweden, Finland, Norway and Switzerland. Birds with an intact beak, however, produce more feather damage and mortality. Without the possibility of beak trimming, other tools are needed to reduce the damage, preferably by reducing the behavior itself and not by just reducing the harmful consequences. Moreover, conventional battery cages in Europe have been prohibited from 2012 onwards (Rodenburg et al., 2012). Now most laying hens are housed in large groups in furnished cages or in barn or free-range systems (non-cage systems) that result in more SFP than before when birds were housed with only four cage-mates in a battery cage (Allen and Perry, 1975). Indeed, large outbreaks of SFP and cannibalism have been recorded in non-cage systems (Appleby and Hughes, 1991; Gunnarsson et al., 1999). Altogether, there is general consensus that due to the larger groups of laying hens and the sharper intact beaks, the risk of severe feather pecking and cannibalism

will increase substantially. In order to solve this problem, unraveling the causal mechanisms of SFP in laying hens has become more important than ever.

PROJECT "PREVENTING FEATHER PECKING IN LAYING HENS: FROM PRINCIPLE TO PRACTICE"

SFP is a multifactorial problem and there is no simple single solution to prevent it. Forty years of research have shown that both genes (nature) and environment (nurture) play an important role in the development of SFP (Hughes and Duncan, 1972; Leonard et al., 1995; Nicol et al., 2001; Huber-Eicher and Audige, 1999; Blokhuis, 1989; Nørgaard-Nielsen et al., 1993). SFP has been suggested to develop from either redirected food pecking (Wennrich, 1974); redirected ground pecking (Blokhuis, 1986); the inability to perform dust bathing (Vestergaard et al., 1993), or redirected social pecking (Riedstra and Groothuis, 2002). Furthermore, severe feather peckers seem to have increased levels of stress (Rodenburg et al., 2009; Rodenburg et al., 2009), fear (de Haas et al., 2012; Hocking et al., 2001; Jensen et al., 2005; Rodenburg et al., 2004; de Haas et al., 2013), sometimes show more aggression (Bessei et al., 2013; Cheng and Muir, 2007) and are more likely to display a proactive coping style (Korte et al., 1997; Koolhaas et al., 1999; Cockrem, 2007). Unfortunately, none of these characterizations of SFP or feather peckers completely explains the origin of this detrimental behavior. It is supposed that the better one understands the causal factors underlying SFP, the better it can be treated and possibly prevented.

Four years ago, the universities of Utrecht, Wageningen, and Groningen started the project "Preventing feather pecking in laying hens: from principle to practice". My research was part of this larger project. The main goal of the larger project was to find predictors and indicators of FP in both experimental and commercially housed chickens at different developmental phases. The impact of both external (e.g. housing conditions and management) and internal (e.g. maternal hormones and neurochemical brain characteristics) factors on the development of SFP behavior have been addressed by two PhD-students and one post-doctoral researcher, focusing on different steps in the production chain (i.e. parent stock, rearing phase, and laying phase), see **Fig. 1.1**. This thesis focuses on the neurobiology of feather pecking and especially the role of the central monoaminergic system; this will be explained below.

THE NEUROBIOLOGY OF FEATHER PECKING BEHAVIOR

Why does abnormal behavior such as SFP occur? This is not an easy question. In humans, behavioral disorders that resemble SFP have been observed, such as body-focused repetitive (constant and recurrent) disorders, for instance, the irresistible urges to pull out hair (Trichotillomania) or to pick at skin (Excoriation disorder) (American Psychiatric Association, 1994). These maladaptive behaviors are often

interpreted by psychiatrists to reflect obsessive-compulsive disorders (OCD) or impulsive-like disorders. It has been hypothesized that central monoaminergic systems (serotonin and dopamine) are involved in SFP (birds) and other compulsive-like behaviors (mammals) (van Hierden et al., 2004; van Zeeland et al., 2009; Dufour et al., 2010). Serotonin (5-hydroxytryptamine; 5-HT) plays a role in, amongst others, regulation of mood, reproduction and growth, impulsivity and aggressiveness (Angoa-Pérez et al., 2012; Dalley and Roiser, 2012; Stein et al., 1991; De Boer et al., 2009; Rabii et al., 1981) whereas dopamine (DA) is involved in motivational and reward-related behavior, motor control, and also higher cognitive functions (Kalenscher et al., 2006). Abnormal behaviors have been related to depletion of the serotonergic and dopaminergic systems. For instance, mice lacking the gene encoding for brain tryptophan hydroxylase 2 - resulting in 5-HT depletion - exhibit more compulsive behaviors, like excessive burying, aggressive and motor impulsive behaviors (Angoa-Pérez et al., 2012).

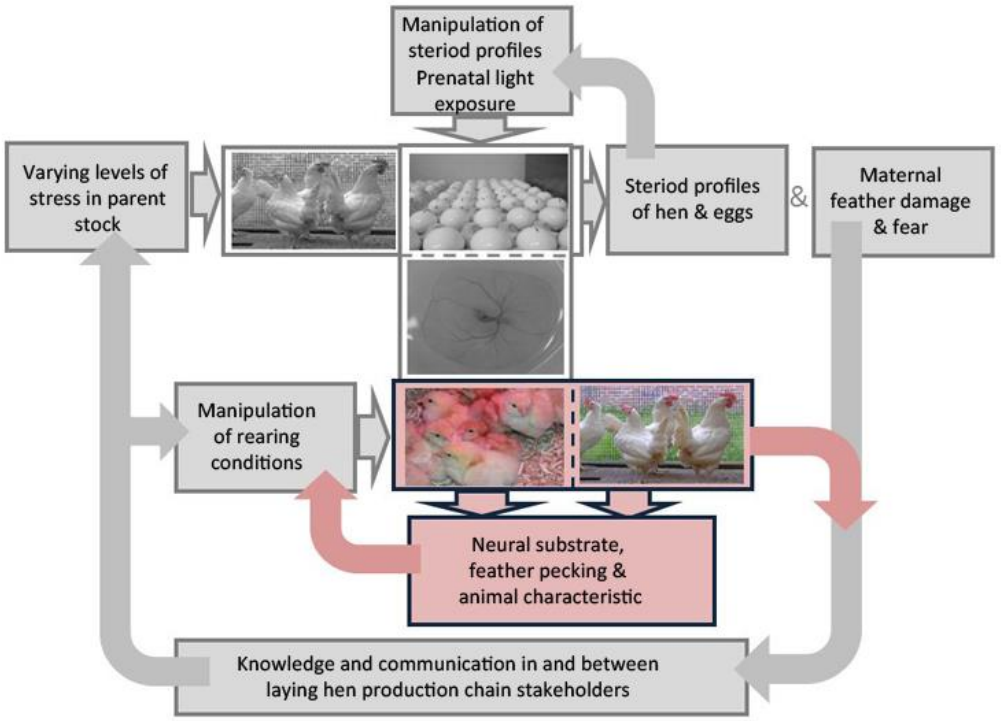

Fig. 1.1 Schematic overview of the projects within project 'Preventing feather pecking in laying hens: from principles to practice'. Figure shows the projects with the different phases in the laying hen production chain, the manipulations that will be applied, the indicators that will be studied in the different projects, and the implementation in practice. The topic of this thesis is depicted in pink. The brains and behavior were studied at both a young and adult age. (Figure adjusted with permission of Dr. T.B. Rodenburg).

Conversely, parrots treated with haloperidol – a dopamine D2 receptor antagonist known to reduce dopamine release– show less feather picking behavior (Iglauer and Rasim, 1993).Similarly, haloperidol treatment has been demonstrated to reduce SFP in adult chickens (Kjaer et al., 2004). Moreover, a chronic dietary supplementation of the 5-HT precursor tryptophan, leading to enhanced 5-HT neurotransmission, decreased SFP in young chickens (van Hierden et al., 2004), whereas the 5-HT_{1A} autoreceptor agonist S-15535, inhibiting 5-HT release, increased the incidence of SFP in young chicks (van Hierden et al., 2005) and adult hens (Dennis et al., 2008a). Thus, there are indications that lower serotonergic and dopaminergic activities are associated with the prevalence of SFP (van Zeeland et al., 2009). The involvement of particular brain areas in the development of SFP is unknown, however, as is the neurobiological profile determining the vulnerability of individual hens developing into a severe feather pecker. These points are addressed in this thesis.

VULNERABILITY TO BECOME A FEATHER PECKER

Both external factors such as rearing and housing conditions (Rodenburg et al., 2008; Johnsen et al., 1998) and diet (van Krimpen et al., 2011; Van Krimpen et al., 2005), but also internal genetic factors contribute to the development of SFP. Heritability of SFP behavior is estimated to range from 0.05 to 0.17 (Su et al., 2005; Rodenburg and Koene, 2003; Kjaer and Sørensen, 1997; Bessei, 1984). Several genetic studies have linked polymorphisms in genes related to the serotonergic and dopaminergic system to SFP behavior (Biscarini et al., 2010; Uitdehaag et al., 2011; Labouriau et al., 2009; Wysocki et al., 2010; Wysocki et al., 2013; Flisikowski et al., 2009). This may suggest that animals with a particular make up and reactivity of monoaminergic systems are prone to develop SFP behavior. Stressful events during the rearing phase, especially, might affect these vulnerable animals. Notably, stressful life-events such as (extreme) aversive environmental conditions during early rearing have a major impact on behavioral and neurological characteristics, such as fearfulness and serotonergic activity (de Haas et al., 2013; Rodenburg et al., 2013). Also, the social environment (like fearful group members) has a clear impact (de Haas et al., 2012). Can we predict which individual young chicken will become a severe feather pecker later in life? This is difficult, because SFP is particularly prevalent in adult hens – at the onset of lay (Uitdehaag et al., 2011; Gilani et al., 2013; Newberry et al., 2007), whereas FP observed in young chicks from commercial lines is mostly gentle by nature. In addition, GFP is shown by the vast majority of young birds, but only a small proportion of the birds develop SFP (Hocking et al., 2001; Rodenburg et al., 2013; Rodenburg et al., 2004; Jones et al., 1995) and, importantly, gentle pecking when young is not predictive for severe pecking as adult (Rodenburg et al., 2013; Rodenburg et al., 2004). This shows the importance of observations of feather pecking at the level of the individual bird, thus the phenotypic characterization of chickens. In commercial practice, however, SFP is often studied indirectly by looking at the prevalence of

feather damage at group level (Bestman et al., 2009; Sherwin et al., 2010). This might be a good indicator for the presence of feather peckers in a group, however, by singling out victims instead of feather peckers no information is gathered on the neurobiological mechanism underlying SFP itself.
Another important factor to consider is brain maturation and brain x behavior interactions. That is, behavior and brain change as manifestations of development that occur in relation to each other (Rogers, 1995). The maturation of a chick's brain continues until ten weeks of age (Rogers, 1995; Atkinson et al., 2008). During that period most important neural connections are established although the brain still has high levels of neural plasticity post-maturation (Rogers, 1995; McEwen et al., 1991; McEwen, 2000). Consequently, if indeed severe feather peckers display lower serotonergic and dopaminergic activity, it is likely that these chickens will adjust their behavior accordingly. Since most previous brain studies focused on young chickens, which, moreover, display mainly GFP and little SFP (e.g. van Hierden et al. 2002), the role of 5-HT and DA in SFP in adult chickens is not clear and both behavior and brain neurochemistry should be studied at different ages.

HOW TO STUDY THE BRAIN OF A FEATHER PECKER?

Brain tissue analyses

Box 1.1 describes the process of the synthesis and metabolism of serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) in the brain. A good method of measuring brain monoamines and their metabolites is by analyzing brain tissue samples. In this way, monoaminergic functioning can be measured in multiple brain areas at once giving insight in the possible role of brain areas with different functionalities. Previous neurological studies showed that low turnover of 5-HT and DA were found in the rostral frontal brain area of young chicks of lines selected on high productivity but also showing high FP incidence in comparison to the low productivity line (van Hierden et al., 2004; Van Hierden et al., 2002). This provided an indication of the involvement of 5-HT and DA in FP, but without discriminating between different brain areas with separate functions. As both 5-HT and DA play an active role in emotional and motor guiding behaviors (Alam et al., 2011; Denbow et al., 1982; Denbow et al., 1981; Gruss and Braun, 1997; Karakuyu et al., 2007), differentiation between brain areas with strong serotonergic and dopaminergic involvement seems a logical step. In this thesis we focused on a maximum of seven brain areas: the dorsal thalamus, a midbrain (diencephalic) area, and six forebrain (telencephalic) areas, the medial striatum (MSt), the hippocampus, the caudolateral nidopallium (NCL) and the caudocentral nidopallium (NCC), the arcopallium and the amygdala. These brain areas are involved in emotional and/or somatosensory regulation with either or both strong serotonergic and/or dopaminergic innervations (Durstewitz et al., 1999b; van den Heuvel et al., 2010; Cai et al., 2011; Jarvis et al.,

2005; Reiner et al., 2004; Güntürkün, 2005; Shanahan et al., 2013; Atoji and Ishiguro, 2009; Metzger et al., 1998; Kröner and Güntürkün, 1999; Cheng et al., 1999; Yamamoto et al., 2005). It is important to realize that this so-called 'punch' method measures a combination of stored and released monoamines and their metabolites (see **Box 1.1**) at a single time point in brain tissue samples, but provides no information on the actual release of neurotransmitters.

In vivo brain microdialysis

In contrast to the post mortem brain tissue analyses described above, microdialysis offers direct measurements of the presynaptic released monoamines at several time points in a limited number of brain areas (usually one, and sometimes two) while the animal is conscious and able to move freely (Westerink, 1995; Benveniste and Hüttemeier, 1990; Ungerstedt et al., 1982). Only after being released from the pre-synaptic neuron 5-HT and DA can act as neurotransmitters and trigger effective signaling between neuronal cells thereby guiding behavioral output (Kandel et al., 2000). The wide-spread use of the microdialysis technique shows the value of this technique in explaining complex neurobiological and neurochemical processes in relation to behavior (Benveniste and Hüttemeier, 1990; Shippenberg and Thompson, 2001). Although microdialysis studies are performed in young chickens (e.g. (Zachar et al., 2012; Tachibana et al., 2000; Gruss et al., 1999; Tsukada et al., 1999), we could not find any published data on microdialysis in adult chickens or on microdialysis in relation with the topic of feather pecking. Apart from brain tissue analyses showing 5-HT and DA involvement in FP, there is a need to investigate the actual release of monoamines as this directly affects behavior.

SELECTION FOR FEATHER PECKING AND OTHER TRAITS

What type of chickens should we study? We already stated the importance of (individual) phenotypic characterization of chickens, but realize this is very time consuming and not very applicable in practice. Genetic selection lines, however, might reveal certain trait-specific differences in the serotonergic and dopaminergic system between high and low feather peckers. In this thesis, we compared behavior and neurobiology of birds from different selection lines. Firstly, a comparison was made between a line selected on productivity and survival of group members, the low mortality line (LML) and a control line (CL) from the same White Leghorn (WL) origin selected on productivity only (Bijma et al., 2007a; Bijma and Wade, 2008; Ellen et al., 2008; Ellen et al., 2010). By selecting against mortality, which is in these non-beak-trimmed birds often a consequence of SFP and cannibalism, the LML may be indirectly selected on low levels of SFP and cannibalism. Behaviorally, the LML showed less cannibalistic toe and comb pecking in the third generation and displayed less fear-related behavior – mostly reflected by more active behavior – compared to

the CL hens in several behavioral tests, both at a young and adult age (Rodenburg et al., 2009; de Haas et al., 2012; Bolhuis et al., 2009; Nordquist et al., 2012). Secondly, a comparison was made between lines divergently selected on SFP behavior with each generation lines differing more in SFP, resulting in the high feather pecking (HFP) line and low feather pecking (LFP) line (Su et al., 2005; Kjaer and Sørensen, 1997; Kjaer et al., 2001). The HFP did not only display more SFP at young and adult age (Bessei et al., 2013), but were also more motivated to eat feathers (Harlander-Matauschek and Feise, 2009) – a behavior related to FP (Rodenburg et al., 2013; Bennewitz et al., in prep.) – had increased locomotor activity in their home cage and were more active in novel environments compared to LFP (Kjaer, 2009; de Haas et al., 2010), although lines did not differ in tonic immobility (Rodenburg et al., 2010). In literature more studies mention using HFP and LFP lines, but it is important to discriminate between lines selected specifically on feather pecking behavior rather than lines that have been selected on other traits, such as productivity, and coincidently diverge in feather pecking tendencies (van Hierden et al., 2005; Van Hierden et al., 2002; Rodenburg et al., 2004) or aggression (Dennis et al., 2008b; Cheng et al., 2001b).

AIM AND SCOPE OF THIS THESIS

The overall objective of this thesis was to further unravel the involvement of serotonergic and dopaminergic signaling in different brain areas in the development of feather pecking behavior. Considering that an animal's internal state is reflected by its behavior and both behavior and brain neurobiology change over time, we focused on the following research questions: 1) what is the effect of (feather pecking) phenotype and/or genotype on the serotonergic and dopaminergic system? 2) Do young and adult high and low feather peckers differ in monoaminergic functioning in different brain areas? 3) Finally, is microdialysis feasible in adult chickens, and if so, do feather peckers differ from non-feather peckers in monoaminergic release?

To address these questions, we combined behavioral observations and tests in individual chickens with the two brain analysis techniques described above. In **Chapter 2**, we focused on adult feather pecking phenotypes. Brain tissues of different brain areas of adult chickens characterized as severe feather peckers, victims or non-peckers were analyzed and compared between phenotypes. In **Chapter 3**, by brain tissue analyses similar to Chapter 2, a comparison was made between adult chickens originating from the LML and CL. **Chapter 4** focuses on young and adult chickens genetically selected on FP behavior, the HFP and LFP. We combined behavioral tests and FP observations with brain research (analysis of seven brain areas) during the rearing phase and the early laying phase. It was expected that the divergence in FP behavior is mirrored by strong monoaminergic differences in the brains of hens from these lines. Since microdialysis provides valuable information on the biological effect of monoamines and our lab is very experienced in microdialysis in rats and mice, we aimed to perform microdialysis in adult chickens. **Chapter 5** describes the

adjustments made to the microdialysis system to fit in larger chickens. In **Chapter 6**, the microdialysis protocol developed was used in adult chickens of the HFP and LFP line. We focused on the nidopallium, a brain area selected based on the results of Chapter 3 and Chapter 4. This brain research was combined with peripheral measurement of tryptophan in blood plasma. Similar to Chapter 4, it was expected that divergent genetic selection with respect to SFP would affect serotonergic and dopaminergic release. The major findings of Chapter 2 to 6 are described and discussed in **Chapter 7**, the general discussion.

BOX 1.1. Synthesis and metabolism of serotonin and dopamine

Serotonin (5-hydroxytryptamine, 5-HT) is synthesized in brain neurons from the essential amino acid L-tryptophan coming from food (referred to as tryptophan in this thesis) (Eisenhofer et al., 2004; Moja et al., 1989). This process takes place in clustered cell bodies of serotonergic neurons in the brain in an area called the raphe nuclei present in both mammals and chickens (Filipenko et al., 2002; Okado et al., 1992; Okado et al., 1989). Dopamine (DA) is synthesized from the non-essential amino acid L-tyrosine (referred to as tyrosine in this thesis) in the ventral tegmental area (VTA) (Moons et al., 1994; Daubner et al., 2011; Fernstrom and Fernstrom, 2007). The production of 5-HT and DA is rate-limited by the activity of the enzymes tryptophan hydroxylase and tyrosine hydroxylase, respectively. Both the raphe nuclei and the VTA project to most parts of the forebrain. There, local serotonergic and dopaminergic nerve endings contain storage vesicles for either 5-HT or DA. When released into the synaptic cleft, these monoamines function as neurotransmitter exciting pre- and postsynaptic receptors corresponding with adequate stimulation or inhibition of second messenger systems, target organs, and/or influence brain areas responsible for a variety of physiologic functions. 5-HT and DA can be metabolized by monoamine oxidase-A enzymes (MAO-A; targeting both 5-HT and DA) and monoamine oxidase-B (MAO-B; targeting DA), although monoamines can be recycled as well. Recycling is mediated by serotonin transporters (SERT) and dopamine transporters (DAT) on the presynaptic neuron. These transporters are responsible for the uptake of the precious monoamines in order to be restored in the presynaptic vesicles for future release. It is a general misconception that MAO-A and MAO-B located in the presynaptic neurons are the largest contributor to metabolites in neural tissue (review by (Youdim et al., 2006). Although dopaminergic neurons contain MAO-A that may target the DA recycled or leaked from the vesicles (review by (Eisenhofer et al., 2004), MAO-B is found in serotonergic neurons (Nagatsu, 2004; Westlund et al., 1988). Probably, this MAO-B eliminates foreign amines competing with 5-HT molecules entering the vesicles (as suggested by (Youdim et al., 2006). Excess 5-HT release in the presynaptic cleft is therefore, just like most DA, metabolized by astrocytes and glial cells containing both MAO-A and MAO-B and also catechol-O-methyl transferase (COMT) (Youdim et al., 2006;

Kimelberg and Katz, 1985). 5-HT is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by MAO-A; DA is metabolized to 3, 4-dihydroxyphenylacetic acid (DOPAC) by MAO-A and MAO-B, but can also be metabolized to 3-methoxytyramine (3-MT) by COMT (review by (Eisenhofer et al., 2004). Both these DA-metabolites are metabolized to homovanillic acid (HVA), the end-product of DA catabolism. Serotonin turnover (5-HIAA/5-HT) and dopamine turnover (DOPAC+HVA+3-MT)/DA) are often calculated as an indirect index for the activity of the serotonergic and dopaminergic system (Korte-Bouws et al., 1996; Hallman and Jonsson, 1984); high turnover ratios indicate higher neuronal activity leading to enhanced release of the neurotransmitter and higher levels of metabolites.

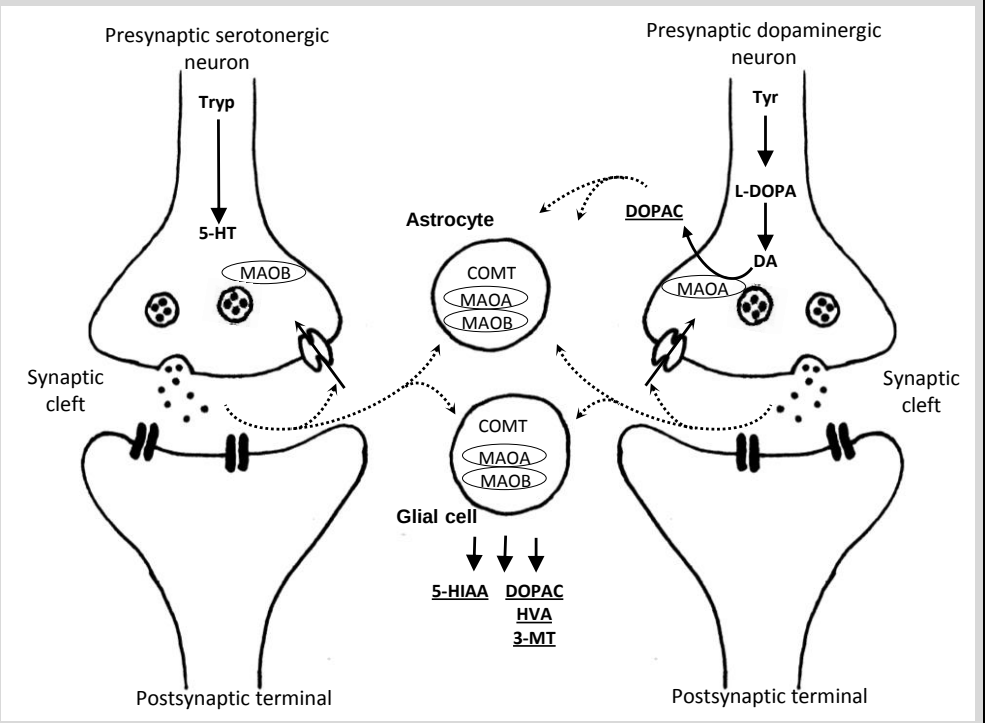

Fig. 1.2 Serotonergic and dopaminergic signaling and metabolism.

Chapter 2

Effects of feather pecking phenotype (severe feather peckers, victims and non-peckers) on serotonergic and dopaminergic activity in four brain areas of laying hens (*Gallus gallus domesticus*)

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ABSTRACT

Severe feather pecking (SFP) in laying hens is a detrimental behavior causing loss of feathers, skin damage and cannibalism. Previously, we have associated changes in frontal brain serotonin (5-HT) turnover and dopamine (DA) turnover with alterations in feather pecking behavior in young pullets (28-60 days). Here, brain monoamine levels were measured in adult laying hens; focusing on four brain areas that are involved in emotional behavior or are part of the basal ganglia-thalamopallial circuit, which is involved in obsessive compulsive disorders. Three behavioral phenotypes were studied: Severe Feather Peckers (SFPs), Victims of SFP, and Non-Peckers (NPs). Hens (33 weeks old) were sacrificed after a 5-min manual restraint test. SFPs had higher 5-HIAA levels and a higher serotonin turnover (5-HIAA/5-HT) in the dorsal thalamus than NPs, with intermediate levels in victims. NPs had higher 5-HT levels in the medial striatum than victims, with levels of SFPs in between. 5-HT turnover did not differ between phenotypes in medial striatum, arcopallium and hippocampus. DA turnover was not affected by feather pecking phenotype. These findings indicate that serotonergic neurotransmission in the dorsal thalamus and striatum of adult laying hens depends on differences in behavioral feather pecking phenotype, with, compared to non-pecking hens, changes in both SFP and their victims. Further identification of different SFP phenotypes is needed to elucidate the role of brain monoamines in SFP.

INTRODUCTION

Severe feather pecking (SFP) is a detrimental behavior in laying hens which leads to feather and skin damage and sometimes even death of the recipient due to cannibalism (Savory, 1995). In search of factors playing a role in increasing the vulnerability for developing into a feather pecker, brain monoamines have become of increasing interest. Several studies investigating the neurobiology of laying hens with high-and low- feather-pecking (FP) incidence, indicated that SFP is linked to lowered serotonergic and dopaminergic turnover (van Hierden et al., 2002; van Hierden et al., 2004; Bordnick et al., 1994; Kjaer et al., 2004; van Hierden et al., 2005; Biscarini et al., 2010; Flisikowski et al., 2009). For instance, young pullets from a high FP line selected for large egg size and good egg-shell qualities showed lower serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) turnover ratios in the rostral part of the forebrain than young pullets from a control line. Also, a 5-HT_{1A} autoreceptor agonist, which inhibits 5-HT release increased the incidence of SFP (van Hierden et al., 2005; Dennis et al., 2008a), while SFP decreased after chronic dietary supplementation of the 5-HT precursor tryptophan (van Hierden et al., 2004). This high FP line also had a more sensitive DA system, as demonstrated by an increased behavioral response to the DA receptor agonist apomorphine (van Hierden et al., 2005). Pharmacological studies showed that treatment with the DA D_2-receptor antagonist haloperidol reduced feather pecking in adult White Leghorns (Kjaer et al., 2004) and feather picking in grey parrots (Iglauer and Rasim, 1993). Thus, FP might be associated with low serotoninergic and dopaminergic neurotransmission. It is important to keep in mind that most studies on 5-HT and DA involvement in FP behavior have been performed in young chicks. These pullets, however, mainly show gentle FP behavior, while SFP behavior usually becomes more pronounced at a later age (van Hierden et al., 2002; Rodenburg et al., 2004). Gentle FP behavior at a young age, however, is not necessarily predictive for SFP behavior in the same individual at adult life (Newberry et al., 2007). Remarkably, only a few animals within a flock will start with SFP, while the majority of birds do not (Keeling, 1994).

SFP is a very persistent and goal-directed behavior with clear impulsive compulsive characteristics (Garner, 2005). Feather damaging behavior also can be seen in parrots (van Zeeland et al., 2009). In mice, a similar type of maladaptive behavior, i.e. barbering, can be observed (Garner et al., 2004), and in humans, a specific hair-pulling disorder, i.e. trichotillomania, is known (Stein et al., 2010; Chamberlain et al., 2009). Both impulse control disorders (ICD) and obsessive compulsive disorders (OCD) in humans are associated with lower levels of brain 5-HT (Fineberg et al., 2010). Mice lacking the gene encoding for brain tryptophan hydroxylase 2 - resulting in 5-HT depletion - exhibit more compulsive behaviors, like burying, aggressive and motor impulsive behaviors (Angoa-Pérez et al., 2012). Deficits and lesions in human frontal-striatal-thalamic brain areas also decrease 5-HT levels and increase the risk for ICD or OCD (Fineberg et al., 2010; Insel et al., 1985; Pattij and Vanderschuren, 2008). It is unknown whether similar deficits in basal ganglia-

thalamopallial circuits underpin SFP in laying hens. Evidence demonstrating functional and structural similarities between avian and mammalian brains is accumulating (Reiner et al., 2004; Yamamoto et al., 2005; Moorman et al., 2012; Herold et al., 2011a; Herold et al., 2012a), therefore it becomes necessary to focus on specific brain areas, instead of entire frontal brains as in previous studies (van Hierden et al., 2002; van Hierden et al., 2004).

In the present study, four brain areas were selected that may be involved in emotional behavior or are part of the basal ganglia-thalamopallial circuit which is involved in obsessive compulsive disorders (OCDs): the telencephalic medial striatum, hippocampus, arcopallium, and the diencephalic dorsal thalamus. The avian ventral striatum, a combination of the medial striatum and the nucleus accumbens, plays an important role in reward (Aoki et al., 2006), as does the human caudate nucleus (Cai et al., 2011). The hippocampus of both mammals and birds seem to be involved in memory and learning (Colombo and Broadbent, 2000). The arcopallium is a somatomotor region surrounded by the subnuclei that constitutes the amygdala (Reiner et al., 2004; Cheng et al., 1999; Yamamoto et al., 2005). Functionally, the arcopallium seems also to be involved in anxiety (Saint-Dizier et al., 2009). Anxiety often forms the base of obsessions and attempts to relieve this anxiety might lead to compulsions (van den Heuvel et al., 2010; Fineberg et al., 2010; Huey et al., 2008). The dorsal thalamus has direct connections with the telencephalic areas (Durstewitz et al., 1999b) and disinhibition of the thalamus will affect goal-directed behavior in humans and animals, with compulsions as risk factor (van den Heuvel et al., 2010).

Measuring 5-HT and DA levels and turnover in these brain areas will increase our knowledge of the neurobiological mechanisms of SFP in laying hens. This study is the first to analyze four brain regions of interest of adult laying hens characterized by their FP phenotype. The phenotypes used are severe feather peckers (SFPs), 'victims', and 'non-feather peckers' (NPs), based on individual observations on giving and receiving SFP. We hypothesize that severe feather peckers show a distinct pattern in monoaminergic levels and turnover, in particular in 5-HT and DA, as compared to the other phenotypes.

MATERIALS AND METHOD

Birds and housing

In this study, 27 female White Leghorns (*Gallus Gallus*) were selected for brain analysis from 260 hens in total of which a subpopulation was previously studied (de Haas et al., 2012). Eighty hens originated from an unselected control line (CL) and hundred and eighty of the fourth generation of a low mortality line (LML) aimed at breeding with those candidates of which siblings showed low group mortality (Ellen et al., 2007). Phenotypes were equally spread among these lines (see Statistical analysis). The hens were obtained from ISA, the layer breeder division of Hendrix Genetics, the

Netherlands. All non-beak trimmed hens were housed per line (26 pens in total, 10 birds /pen) in 1.9 x 1.2 m pens. Water and a commercial mash diet were provided *ad libitum*. Pens were provided with a perch and floors were covered with sand (1/3) and wood shavings (2/3). For more details on housing conditions, see (de Haas et al., 2012). The experiment was approved by the Institutional Care and Use Committee of Wageningen University and in accordance with Dutch legislation on the treatment of experimental animals, in conformation with the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

Categorizing behavioral phenotypes

FP behavior was observed using behavior sampling over three weeks for 30 minutes each week on varying times during the day (9.00am- 16.00am), at an age of 19, 20 and 21 weeks. We recorded the frequency of severe feather pecking (SFP), i.e. bouts of hard pecks and pulling attempts directed at feathers at the tail, back and wings. A bout of SFP was defined as pecks in a continuous series directed to the same chicken to the same body part. Gentle feather pecks (*nibbling* on feathers) and aggressive pecks (directed at head, see (Bilcik and Keeling, 1999) were also recorded, but not taken into account in the categorization procedure. Hens were categorized as feather pecker (SFPs) when giving a minimum of two SFP bouts over the observations weeks, without receiving any SFP bouts, as victim when receiving at least one SFP bout, but not giving SFP; or as non-pecker if not giving or receiving SFP. For brain analysis, we selected n=9 birds per behavioral phenotype. To account for pen effects, the selection of phenotypes was balanced over pens, that is, we chose one SPF bird, one non-pecker and one victim per pen. The three FP phenotypes did not differ in aggressive pecks given or received (data not shown).

Brain tissue preparation

At 33 weeks, chickens were subjected to a 5-min manual restraint test on two consecutive days, using a method previously described (Bolhuis et al., 2009). Order of testing was balanced for phenotype and line. Immediately after testing, the hens were sacrificed by cervical dislocation. Brains were removed and immediately deep frozen in n-heptane, put on dry ice and stored at -80^{o}C (van Hierden et al., 2002). Slicing of brains was executed in a cryostat (Frigocut Jung Mod_700) under cold conditions (-10^{o}C). Slice thickness was 400 μm. The four regions of interest were located using the brain atlas for 2-week-old chickens (Puelles et al., 2007), thereby taking into account the increased brain size in our hens at 33-weeks of age. Punches were taken from multiple slices, with corresponding figure numbers in the atlas: Medial striatum (MSt; interaural 7.56 - 5.68mm) including the accumbens (Acb; interaural 8.08 - 7.56mm), hippocampus (Hi1, Hi2, PHiM, PHil, PHil1, PHil 2, and PHiA; interaural 6.16 - 0.40mm), and the dorsal thalamus (DPe, DMA, DIA, DLA; interaural 3.04 - 1.36mm). For the arcopallium, the area referred to as amygdala core by (Puelles et al., 2007) was

sampled (interaural 4.24 - 2.08 mm). Brain samples of the left and right hemisphere were taken together and analyzed as one.

Central monoamine analysis with HPLC

Brain samples were analyzed using a High Performance Liquid Chromatography (HPLC) method. For that, the tissue samples were homogenized in an ice-cold solution containing 5 µM clorgyline, 5µg/ml glutathione and 1.2 µM N-methylserotonin (NMET, internal standard) using sonication. To 80 µl homogenate, 20 µl 2 M $HClO_4$ was added and mixed. After 15 min in ice water, the homogenates were centrifuged for 15 min at 15000g (4 °C). The supernatants were diluted 10 times with water before HPLC analysis. The concentration of serotonin [5-HT] and its metabolite 5-hydroxyindoleacetic acid [5-HIAA], and dopamine [DA] with according metabolites 3-methoxytyramine [3-MT], 3, 4-dihydroxyphenylacetic acid [DOPAC], and homovanillic acid [HVA] in the tissue extracts were measured by HPLC with electro chemical detection (ECD). The HPLC system consisted of a pump model P100, a model AS300 autosampler (both from Thermo Separation Products, Waltham, MA, USA), a ERC-3113 degasser (Erma CR. Inc. Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +550mV (ESA Inc. Bedford MA, USA), a data acquisition program (Atlas 2003, Thermo Separation Products) and a column (150mm x 4.6mm i.d.) packed with Hypersil BDS C18, 5 µm particle size (Alltech Associates, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µl/L dibutylamine, 77 mg/L 1-octanesulfonic acid sodium salt, 10 % methanol; the pH of the buffer was adjusted to 3.4 with NaOH. Separation was performed at 45 °C using a flow rate of 0.8 ml/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The protein content of each homogenate sample was determined using the DC protein Assay (Bio-Rad). Monoamine concentrations are expressed as nmol/g protein. Turnover ratios of serotonin (5-HIAA/5-HT) and dopamine ((DOPAC+HVA+3-MT)/DA) were calculated as an index for the activity of, respectively, the serotonergic and dopaminergic system (van Hierden et al., 2002); high levels indicate a quicker metabolic pathway due to higher biosynthetic enzyme activity.

Statistical analysis

Effects of phenotype (SFPs vs. victims vs. NPs) on monoamines were analyzed with a general linear model (GLM) that included effects of line (C and L) and day of sacrifice, using SAS Software 9.2. In a preliminary analysis of the data, no interactions between phenotype and line were found. In case of significant phenotype effects, post-hoc least square means were used to detect pair-wise differences. A log transformation for 5-HT

and 5-HIAA in the hippocampus was executed to obtain normality of residuals. Data are presented as mean ± SEM.

RESULTS

Feather pecking phenotypes

In nine out of 26 pens SFP was detected during observations. This led to the categorization of the 'SFPs', 'victims', and 'NPs' phenotypes (n=9 /phenotype). The SFP hens selected gave on average 3.4 ± 0.5 bouts of SFP and received none, victims received on average 2.1 ± 0.4 bouts of SFP and gave none, and NPs gave zero bouts of SFP, but one out of the nine NP-characterized hens received one bout of SFP (average 0.1 ± 0.1).

Phenotype effects on dopamine

In the hippocampus, levels of DA and of its metabolites were below detection level for most samples. In the other brain areas, phenotype did not affect DA, DOPAC, HVA, and 3-MT levels (data not shown), except that HVA ($F_{2,22}$ = 3.63, P<0.05) in the arcopallium was higher in SFPs (18.1 ± 2.1) and tended to be higher in victims (18.1 ± 3.0) in comparison to NPs (13.1 ± 2.0), without affecting DA turnover. DA turnover did not differ between the phenotypes (dorsal thalamus: P>0.10, 1.17 ± 0.08; medial striatum: P>0.10, 0.24 ± 0.01; arcopallium: P>0.10, 1.45 ± 0.17).

Phenotype effects on serotonin

5-HT levels in the dorsal thalamus (**Fig. 2.1A**) were unaffected by phenotype, but phenotype affected 5-HIAA levels in this brain area ($F_{2,21}$ = 5.43, P<0.05) with SFPs having higher levels than NPs (P<0.01). Thalamus 5-HIAA levels of victims were intermediate between SFP and NPs, but tended to be higher than those of NPs (P<0.10). Phenotypes differed in 5-HT turnover in the dorsal thalamus ($F_{2,21}$ = 5.67, P<0.05). Post-hoc tests revealed that SFPs and victims both had a higher 5-HT turnover than NPs.

In the medial striatum (**Fig. 2.1B**), phenotype did affect 5-HT levels ($F_{2,22}$ = 4.72, P<0.05). Post-hoc tests indicated higher 5-HT levels for NPs compared to victims, with intermediate levels in SFPs. 5-HIAA in the medial striatum tended to be affected by phenotype ($F_{2,22}$ = 2.89; P<0.10), with higher levels for SFPs than for victims and a tendency for higher levels in NPs than in victims. 5-HT turnover in the medial striatum was unaffected by phenotype.

In the arcopallium and hippocampus, no significant effects of phenotype on 5-HT, 5-HIAA or 5-HT turnover were found (**Table 2.1**).

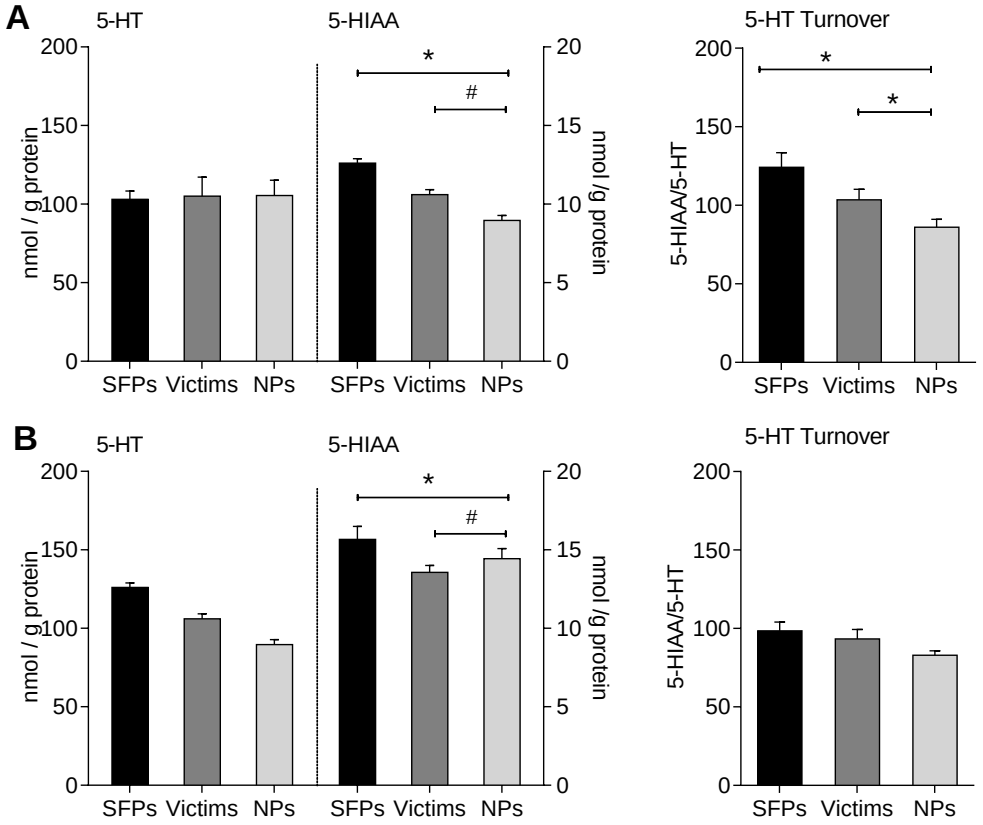

Fig. 2.1 Levels of 5-HT and 5-HIAA (in nmol/g protein), and 5-HT turnover in the dorsal thalamus (A) and medial striatum (B) of laying hens characterized as severe feather peckers (SFPs), victims, or non-featherpeckers (NPs), *n* = 9 per phenotype, **P*<0.05, # *P*<0.10

Table 2.1 5-HT and 5-HIAA levels* and 5-HT turnover in the arcopallium and the hippocampus of hens characterized as severe feather peckers (SPFs), victims, and non-feather peckers (NPs)

	SFPs	Victims	NPs	*P*-value
Arcopallium				
5-HT	247.2 ± 23.8	287.9 ± 43.4	241.3 ± 32.0	0.52
5-HIAA	25.8 ± 1.7	27.3 ± 3.6	20.4 ± 2.6	0.13
5-HT turnover	107.8 ± 8.3	98.9 ± 8.0	86.0 ± 6.1	0.44
Hippocampus				
5-HT	156.0 ± 16.4	181.1 ± 27.2	164.9 ± 11.7	0.96
5-HIAA	19.8 ± 3.3	21.0 ± 1.7	21.7 ± 1.4	0.55
5-HT turnover	127.9 ± 19.2	125.9 ± 13.3	131.4 ± 5.7	0.95

*5-HT and 5-HIAA in nmol/g protein, mean ± SEM, *n*=9 / phenotype

DISCUSSION

Monoaminergic turnover was measured in adult laying hens that differed in phenotype: severe feather pecking (SFPs), victims of SFP, and non-feather peckers/non-victims (NPs). The most pronounced findings were: 1) both SFPs and victims had higher 5-HT turnover in the dorsal thalamus as compared to NPs, 2) in the medial striatum, NPs had higher 5-HT than victims with SFPs in between, 3) no phenotypic differences in 5-HT neurotransmission were found in the arcopallium or hippocampus, and 4) DA turnover did not differ between phenotypes in any of the four brain areas.

Dopaminergic activity

There were no dopaminergic differences found between phenotypes, with exception of higher HVA levels for SFPs and victims than for NPs in the arcopallium. This single result shares resemblance with higher DOPAC levels, also a DA metabolite, measured in the rostral part of the brain of adult chickens from the White Leghorn line with more feather damage due to FP in comparison with a Rhode Island Red line low in feather damage (Uitdehaag et al., 2011). Still, the absence of strong dopaminergic phenotypic differences is unexpected as several studies have indicated that modulating dopaminergic activity affects FP behavior (van Hierden et al., 2002; Kjaer et al., 2004; Flisikowski et al., 2009; Cheng and Long, 1974). It has to be noted, though, that due to strong neural and monoaminergic connections, serotonergic abnormalities will affect the dopaminergic system (described by Prins et al., 2011b), thus despite the lack of dopaminergic turnover differences between phenotypes the DA system can still be affected although not shown by the used method (punches) in the present study.

Serotonergic activity

Differences in serotonergic neurotransmission of the different behavioral phenotypes were mainly found in the dorsal thalamus, but not in the medial striatum, hippocampus or arcopallium. In the dorsal thalamus, SFPs and victims had a higher 5-HT turnover than NPs due to higher 5-HIAA levels.

5-HT turnover in the medial striatum, albeit numerically higher for SFPs, did not significantly differ between the three phenotypes, despite significant differences for 5-HT and 5-HIAA separately. That is, NPs had higher 5-HT levels than victims with intermediate levels in SFPs, while for 5-HIAA, SFPs tended to have higher levels than victims with intermediate levels in NPs. In line with other studies (van Hierden et al., 2002; Bordnick et al., 1994; van Hierden et al., 2004; van Hierden et al., 2005; Uitdehaag et al., 2011; Flisikowski et al., 2009), 5-HT neurotransmission is altered in SFPs, but also in their victims.

Stress factors contribute to brain alterations. Captivity in general (Morgan and Tromborg, 2007) and more specifically, group size (Bilcík and Keeling, 2000),

fearfulness of group members (de Haas et al., 2012; Uitdehaag et al., 2008b), housing conditions (Patzke et al., 2009), and the absence of maternal care (Rodenburg et al., 2009) are stress factors that will affect behavior and physiology, including serotonergic structures and their function (Patzke et al., 2009; Cheng and Fahey, 2009). In general, feather peckers seem to differ in stress responsiveness: Differences in basal and stress-induced CORT were found in lines diverging in FP, although both high (Vestergaard et al., 1997; Kjaer and Guemene, 2009) and low (van Hierden et al., 2002; Korte et al., 1997; Koolhaas et al., 1999) levels of CORT have been associated with high SFP incidences. Feather peckers also had a higher heart rate during manual restraint than NPs (Korte et al., 1998) and had altered exploration in an open field (Rodenburg et al., 2004). It is known that thalamic and striatal areas as part of the basal ganglia-thalamopallial circuit, are involved in guiding motor actions and decision-making by integrating sensorimotor, motivational, and emotional information from the cortex and limbic areas (Jarvis et al., 2005; Reiner et al., 2004; Seifert et al., 2011). For instance, low 5-HT levels after lesions in the medial striatum of chickens impair the suppression of immediate reward seeking behavior (Izawa et al., 2003), which is considered impulsiveness. It can be speculated that the altered 5-HT neurotransmission reflects increased vulnerability for stressful events in SFPs with corresponding anxiety and increased risk for developing impulsive/compulsive disorders (van den Heuvel et al., 2010; Fineberg et al., 2010; Huey et al., 2008) causing chickens to develop SFP. In birds, the exact role of 5-HT in these brain areas and the development of OCD and anxiety needs further investigation.

Surprisingly, NPs had a decreased 5-HT turnover in the dorsal thalamus as compared to both SFPs and victims. Interestingly, a gene expression study in abnormal tail biting pigs showed that non-biting pigs differed distinctly from tail-biters and their victims (Brunberg et al., 2013), similar to these results in chickens. This phenotypic distinction is further supported by the different gene expression patterns in feather pecking chickens that are related to OCD (SFPs, victims, and NPs) (Brunberg et al., 2011). The similarity between SFPs and victims might be explained by victims perceiving attacks of SFP as highly unpredictable and stressful. It has been shown that unpredictable tail-shocks in rats increase 5-HIAA and 5-HIAA/5-HT ratio levels in the thalamus and striatum (Adell et al., 1988) and that being victimized may lead to long term structural adaptations in monoaminergic systems (De Kloet et al., 2005). Thus as feather pecking affects both SFPs and victims, NPs seem to remain aloof; showing the importance of phenotypic discrimination.

Our results suggest that both victims and SFPs respond with higher 5-HT activity in the dorsal thalamus. The higher 5-HT turnover ratio in SFPs in this study is seemingly in contrast with literature on impulsive and compulsive disorders in humans, rats and mice (De Boer et al., 2009; Stein et al., 2010; Chamberlain et al., 2009) and in contrast with earlier established negative correlation between 5-HT turnover and time spent on FP behavior in young (28-60 days old) chicks (van Hierden et al., 2002; van Hierden et al., 2004; van Hierden et al., 2004). Previously,

van Hierden and collaborators (van Hierden et al., 2002) showed that high FP young (mostly gently FP) pullets have a much higher responsivity towards serotonergic drugs or tryptophan than low FP pullets. It was shown by de Boer and collaborators (De Boer et al., 2009; Koolhaas et al., 2007) that enhanced inhibitory control of the serotonergic raphe neurons (via 5-HT1A autoreceptor) in trained aggressive male rodents might explain the negative correlation between 5-HIAA/5-HT and aggression. This inverse relationship between tonic (trait-like) 5-HT activity and aggression, however, was only observed in the pathological form of aggression (violence). In young pullets, however, stimulation of the 5-HT1A autoreceptor lowered aggression, but increased the time spent on feather pecking behavior (van Hierden et al., 2004). Thus, the motivational drive and neurobiological mechanism involved in high aggression and severe feather pecking behavior probably really differs.

Remarkably, Uitdehaag and colleagues showed higher 5-HIAA and 5-HT turnover in adult hens of the White Leghorn line that are characterized by high scores of SFP, compared to adult hens of the brown Rhode Island Red line that do not show much SFP (Uitdehaag et al., 2011). Thus, multiple factors may contribute to FP at different moments in life probably due to plasticity of the brain and behavioral interactions between birds (e.g. copying behavior or attraction to damaged feathers). This might increase the vulnerability to develop (mostly gentle-) FP at a young age, while an increased 5-HT turnover as found here (and in Uitdehaag et al., 2011) in adult laying hens may reflect a changed 5-HT signaling in response to being exposed to or executing SFP behavior throughout and later in life (either as victim or as feather pecker). The latter puts emphasis on the importance of separating cause and consequence of FP on brain monoamine levels. Besides indications that FP may arise due to disturbances at a neurochemical level ("cause"), animals can change their own behavior ("consequence") by observing others, called social transmission (Zeltner et al., 2000). Although only the transmission of gentle FP among chickens is confirmed (McAdie and Keeling, 2002), this still asks for carefulness when studying FP phenotypically. That is, if SFP within in a group is started by birds with disrupted monoaminergic levels, but is facilitated in others birds, characterizing phenotypes and relating brain-to-behavior becomes complicated. Altogether, it is important to consider both phenotype (i.e. the composite of an organism's observable characteristics) and genotype (i.e. the genetic contribution to the phenotype) when trying to identify causal mechanisms of FP. Hence, we hypothesize the existence of different phenotypes of feather peckers: 'first order' feather peckers (partly determined by genotype) which may be vulnerable to develop SFP due to (heritable) neurochemical "deficits", and 'second order' feather peckers (mainly determined by environment and thus phenotype) in which the feather pecking is socially facilitated or feather pecking is increased because the bird's attention is drawn to the damaged feathers of the victim. Remarkably, (passively coping) chickens with initially low FP incidences are more vulnerable to show 'second order' feather pecking, because they are more aware of environmental changes (Koolhaas et al., 1999) and/or might

respond to the appearance of damaged or ruffled feathers (McAdie and Keeling, 2000). Therefore, differences in coping strategy or personality may underlie the differences between first and second order feather pecking phenotypes. For instance, proactive and reactive (passive) coping style can be distinguished reflecting different ways of coping with stressful circumstances (reviews by Koolhaas et al., 1999; Korte et al., 2005; Korte et al., 2009) with behavioral and physiological interspecific variations (van Hierden et al., 2002; Korte et al., 1997; Korte et al., 1998; Forkman et al., 2004). Characterizing severe feather peckers as early as possible is the best way to distinguish the different feather pecking phenotypes, e.g. by identifying clear biomarkers with predictive value.

CONCLUSION

To our knowledge, this study is the first to link severe FP behavior in adult laying hens to central 5-HT and DA turnover ratios in four brain areas. Unexpectedly, dopaminergic neurotransmission was not affected by phenotype, but SFPs and their victims did have higher 5-HT neurotransmission in the dorsal thalamus and medial striatum. Therefore, it can be concluded serotonergic activity in the dorsal thalamus and striatum of adult laying hens depends on differences in behavioral phenotype, i.e. severe feather peckers, victims or non-peckers.

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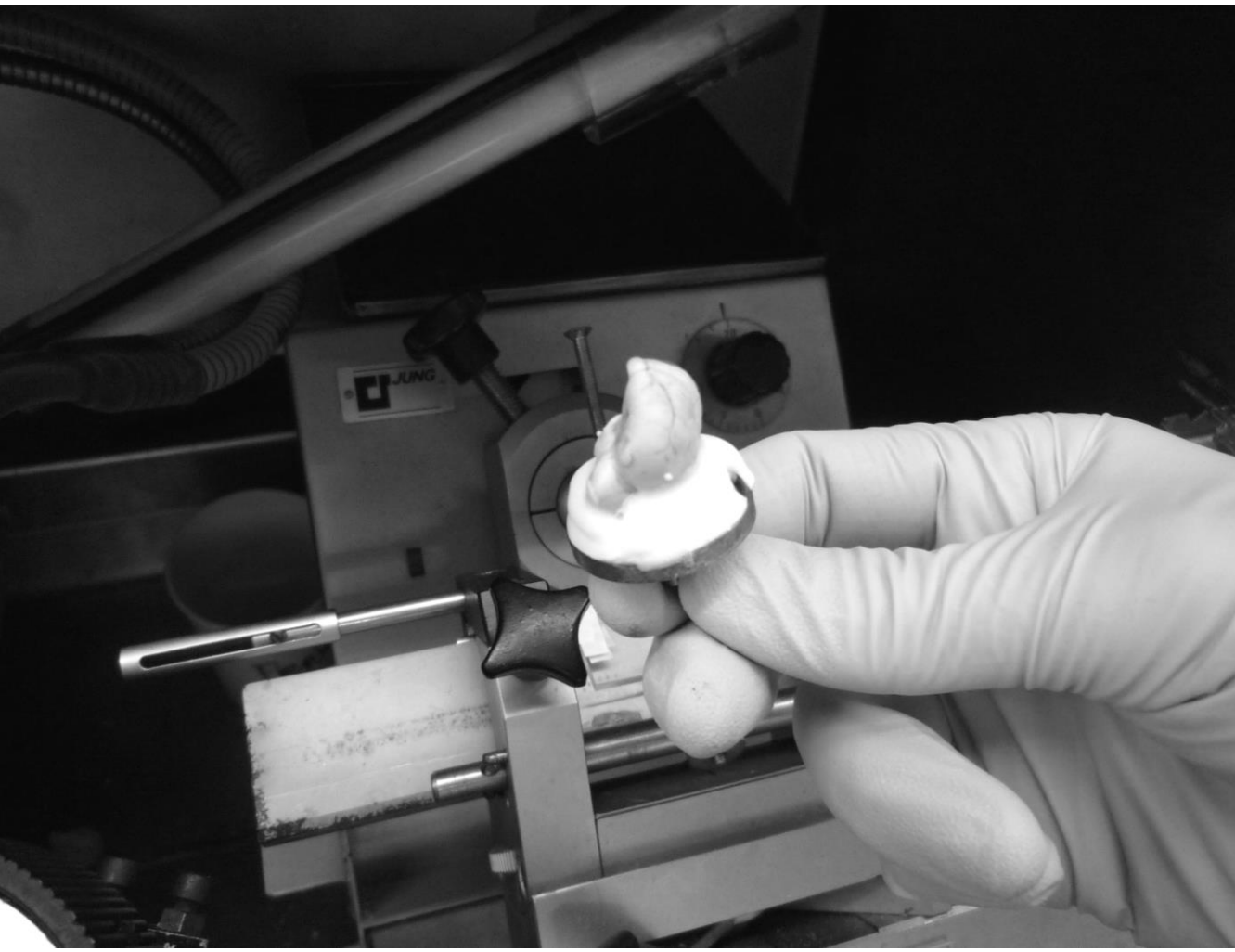
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Chapter 3

Selection for low mortality in laying hens affects catecholamine levels in the arcopallium, a brain area involved in fear and motor regulation

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ABSTRACT

Severe feather pecking (FP) in laying hens may cause mortality due to cannibalism. Novel breeding methods using survival days of group-housed siblings allow for the genetic selection of laying hens with low mortality (LML: low mortality line) due to cannibalism. Previous studies have demonstrated less fear-related behavior and also less FP in LML hens compared to CL. Selection also caused changes in locomotor behavior in an open field. It is unknown, however, whether selection for low mortality affects central neurotransmitter levels. In this study, brain monoamine levels were measured in the dorsal thalamus, medial striatum, hippocampus and arcopallium of adult laying hens of both LML and CL using HPLC. Brain samples were collected after 5-min of manual restraint. The most prominent line differences were found in the arcopallium. Compared to CL, LML had lower levels of noradrenaline (NA) and 3,4-dihydroxyphenylacetic acid (DOPAC) and tended to have lower levels of dopamine (DA), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA). Levels of serotonin (5-HT), 5-HT- and DA-turnover in this brain area were not affected by line. LML showed less fear-related behavior during the restraint than CL. These findings show that selection for low mortality in hens leads to changes of predominantly the dopaminergic system in the chicken's arcopallium, a forebrain somatomotor area also related to fear. This suggests a relationship between catecholamine functioning in this brain area and FP and cannibalistic behavior in chickens and underpins previously found relationships between FP, fear and high activity.

INTRODUCTION

Welfare concerns have led to a European ban on conventional battery cages for laying hens. Since 2012, only alternative housing systems ranging from "furnished" or "enriched" cages to non-caged aviaries or free-range systems are allowed (Rodenburg et al., 2012). Although these systems allow hens more freedom of movement, recent studies report increased mortality rates within alternative housing systems and many casualties are due to cannibalism (Fossum et al., 2009). Cannibalism in laying hens is the act of a bird pecking at the skin and devouring the flesh of other birds (Savory, 1995), which may ultimately lead to the death of the victims. Cannibalistic pecking is often preceded by severe feather pecking (FP) which is pecking at and removing of feathers of conspecifics causing denuded areas in the plumage which subsequently is very attractive for others to peck at (Bilcik and Keeling, 1999). To reduce problems related to severe feather pecking and cannibalism, many hens are exposed to beak-trimming, i.e. removal of the sharp tip of the upper beak. There is a growing societal resistance against animal mutilations, as it in itself beak-trimming induces stress, pain and fear in hens (Gentle, 2011; Gentle et al., 1990). Thus, there is an urgent need for alternatives to reduce severe FP and cannibalism in laying hens.

Multiple factors, such as rearing and housing conditions (Rodenburg et al., 2008; Johnsen et al., 1998), and diet (van Krimpen et al., 2011; Van Krimpen et al., 2005) may contribute to the development of FP and cannibalism in laying hens. There are, however, also large individual differences in the vulnerability to develop severe FP and cannibalism and a genetic background for these differences has been found (Kjaer et al., 2001; Cheng et al., 2001a). Traditionally, laying hens are selected for individual performance, such as egg production (Olsen and Knox, 1940). Selecting on individual performance can have potentially negative side-effects on group members (Bijma et al., 2007a; Bijma et al., 2007b). By focusing on group performance and survival, Craig and Muir successfully decreased cannibalism-induced mortality in non-beak trimmed hens (Craig and Muir, 1996; Muir, 1996). Recently, a novel selection method has been developed in which selection of individually housed candidates is partly based on the survival of their group-housed female siblings (Ellen et al., 2007). The advantage of this selection method is that candidates for breeding remain unaffected by group interactions, as they are housed individually, and vital information on individual performance is combined with the information on group performance.

Already in the first generation a markedly decreased mortality rate was established in the low mortality line (LML) compared to the unselected control line (CL) (Ellen et al., 2010; Rodenburg et al., 2010). In the third generation, LML showed less cannibalistic toe and comb pecking than CL (Rodenburg et al., 2009). Behavioral tests further show that LML hens displayed less fear-related and more active behavior compared to the unselected CL hens in several behavioral tests, both at young and at adult age (Rodenburg et al., 2009; de Haas et al., 2012; Bolhuis et al., 2009; Nordquist et al., 2012). Interestingly, LML and CL hens do not only differ in damaging and emotional behavior, but also in possible underlying physiological mechanisms. For

example, differences in the peripheral serotoninergic system have been found, with LML hens having higher whole blood serotonin (5-hydroxytryptamine; 5-HT) levels (Rodenburg et al., 2009; Bolhuis et al., 2009) and a lower platelet 5-HT uptake (Bolhuis et al., 2009) than CL. Also, lower plasma corticosterone levels were measured in LML after a manual restraint (Rodenburg et al., 2009) possibly reflecting decreased fearfulness in the low mortality line. In animals and humans, anxiety (or fearfulness) has been related to brain 5-HT (Lesch et al., 1996). Several genetic and pharmacological studies further established the involvement of both central 5-HT and DA in FP (van Hierden et al., 2004; Bordnick et al., 1994; Kjaer et al., 2004; van Hierden et al., 2005; Biscarini et al., 2010; Flisikowski et al., 2009) and cannibalism (Rodenburg et al., 2009; Flisikowski et al., 2009; Cheng et al., 2001a; Cheng et al., 2003; Dennis et al., 2006). More recently, a lower concentration of tyrosine hydroxylase, the rate-limiting enzyme in DA production, was reported in the nidopallium, a "prefrontal" area (Güntürkün, 2005; Kröner and Güntürkün, 1999; Mogensen and Divac, 1982) of LML hens compared with CL hens (Nordquist et al., 2013). Unknown, however, is whether and how selection for low mortality in laying hens affects central neurotransmitter levels in the brain.

The aim of the present study was to compare brain monoamine levels and DA and 5-HT turnover ratios between the fourth generation of laying hens selected for low mortality (LML) and the control line (CL). In total, four target regions related to the modulation of fear and motor control were selected (Durstewitz et al., 1999b; Atoji et al., 2006): a combination of the dorsal (AD) and central (AI) region of the intermediate arcopallium (referred to as arcopallium), the medial striatum, the hippocampus, and the dorsal thalamus. The arcopallium receives input from various associative and sensory forebrain areas and is the source of a major down-sweeping pathway to brainstem motor structures; it thus is a somatomotor forebrain area (Reiner et al., 2004). The medial striatum is the limbic component of the avian striatal complex (Reiner et al., 2004). Given the behavioral and physiological differences between LML and CL, we expect lower levels for both DA and 5-HT in CL hens compared to LML hens, as the former are considered more fearful and display more FP and cannibalistic behavior leading with higher mortality rates.

MATERIAL AND METHODS

Ethical statement

The experiment was approved by the Animal Care and Use Committee of Wageningen University, and in accordance with Dutch legislation on the treatment of experimental animals the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

Birds and housing

In total, 40 adult female White Leghorns (*Gallus gallus domesticus*) of 33 weeks of age were selected for brain analyses. Half of these hens originated from CL (n=20) and the other half from the fourth generation of LML (n=20) aimed at breeding with selection candidates of which siblings showed low group mortality (de Haas et al., 2012; Ellen et al., 2007). All non-beak trimmed hens came from the same population of 160 hens as described previously (de Haas et al., 2012) and were obtained from ISA, the layer breeder division of Hendrix Genetics, the Netherlands. Hens were housed per line in groups of 10 birds per pen, 8 pens per line thus 16 pens in total. From each pen, two to three hens were randomly selected for brain analyses such that twenty birds were selected per line. Water and a commercial mash diet were provided ad libitum. Pen floors (1.9 by 1.2 m) were covered with sand (1/3) and wood shavings (2/3). For more details on housing conditions, see (de Haas et al., 2012).

Manual restraint

At 33 weeks, each hen was subjected to a manual restraint test, using a method previously described (Bolhuis et al., 2009). Briefly, each hen was removed from her home pen and put in a cardboard box to be tested in an adjacent room. The experimenter used the right hand to place a hen on her right side on a table covered with cardboard and then covered the trunk and the left hand gently stretched the hen's legs. For 5 min, the frequency of consecutive struggles and the number of vocalizations was recorded as well as the latency to struggle and vocalize. After each struggle, hens were placed back into the original test position until the time of the test passed. The manual restraint took place on two consecutive days by one researcher and the order of testing was balanced for line. After the manual restraint, the hens selected for brain analyses were sacrificed by cervical dislocation.

Brain tissue preparation

Brains were removed and immediately deep frozen in n-heptane, put on dry ice and stored at -80^{o}C (protocol by (van Hierden et al., 2002). Slicing of brains was executed in a cryostat (Frigocut Jung Mod_700) under cold conditions (-10^{o}C). Slice thickness was 400 µm. The four regions of interest were located using the brain atlas for 2-week-old chickens (Puelles et al., 2007) and with considering literature on the avian brain (Metzger et al., 1998; Atoji and Wild, 2009; Metzger et al., 2002), thereby also taking into account the increased brain size in our hens at 33-weeks of age. **Fig. 3.1** is a schematic drawing depicting the location of, respectively, the MSt (A) and the dorsal thalamus, hippocampus and the arcopallium (B). The gray dotted shapes illustrate the cutting lines per brain area (carefully cut with a scalpel). Tissue samples were taken from multiple slices, with corresponding figure numbers in the atlas: Medial striatum (MSt; 7.56 - 5.68 mm anterior to the interaural line) including the accumbens (Acb;

8.08 - 7.56 mm anterior to the interaural line), hippocampus (HIPP; Hi1, Hi2, PHiM, PHil, PHil1, PHil 2, and PHiA; 6.16 - 0.40 mm anterior to the interaural line), and the dorsal thalamus (DPe, DMA, DIA, DLA; 3.04 - 1.36 mm anterior to the interaural line). For the arcopallium, the area referred to as amygdala core by (Puelles et al., 2007) was sampled (4.24 - 2.08 mm anterior to the interaural line). Brain samples of the left and right hemisphere were taken together and analyzed as one.

Central monoamine analysis with HPLC

Brain samples were analyzed using a high performance liquid chromatography (HPLC) method. For that, the tissue samples were homogenized in an ice-cold solution containing 5 µM clorgyline, 5µg/ml glutathione and 1.2 µM N-methylserotonin (NMET, internal standard) using sonication. To 80 µl homogenate, 20 µl 2 M $HClO_4$ was added and mixed. After 15 min in ice water, the homogenates were centrifuged during 15 min at 15.000 x g (4 °C). The supernatants were diluted 10 times with water before HPLC analysis. The concentration of serotonin [5-HT] and its metabolite 5-hydroxyindoleacetic acid [5-HIAA], and dopamine [DA] with corresponding metabolites 3-methoxytyramine [3-MT], 3,4-dihydroxyphenylacetic acid [DOPAC], and homovanillic acid [HVA], and also noradrenaline [NA] in the tissue extracts were measured by HPLC with electro chemical detection (ECD). The HPLC system consisted of a pump model P100, a model AS300 autosampler (both from Thermo Separation Products, Waltham, MA, USA), a ERC-3113 degasser (Erma CR. Inc. Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +550mV (ESA Inc., Bedford MA, USA), a data acquisition program (Atlas 2003, Thermo Separation Products) and a column (150mm x 4.6mm i.d.) packed with Hypersil BDS C18, 5 µm particle size (Alltech Associates, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µl/l dibutylamine, 77 mg/l 1-octanesulfonic acid sodium salt, 10 % methanol; the pH of the buffer was adjusted to 3.4 with NaOH. Separation was performed at 45 °C using a flow rate of 0.8 ml/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The protein content of each homogenate sample was determined using the DC protein Assay (Bio-Rad). Monoamine concentrations are expressed as nmol/g protein. Turnover ratios of serotonin (5-HIAA/5-HT) and dopamine ((DOPAC+HVA+3-MT)/DA) were calculated as an index for the activity of the serotonergic and dopaminergic system (van Hierden et al., 2002); high levels indicate a quicker metabolic pathway due to higher biosynthetic enzyme activity.

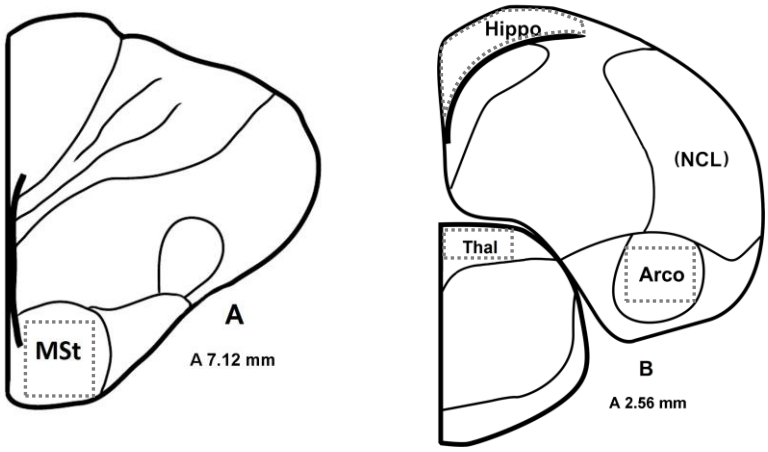

Fig. 3.1 Coronal schematic views of the chicken brain illustrating the medial striatum, thalamus, hippocampus, and arcopallium. The schematic views of the left hemisphere of a chicken's brain are drawn after (Metzger et al., 1998; Atoji and Wild, 2009; Metzger et al., 2002) with brain coordinates based on the chicken brain atlas (Puelles et al., 2007). Depicted are the medial striatum (MSt) at 7.12 mm anterior to the interaural line (A), and the thalamus, arcopallium and hippocampus at 2.56 mm anterior to the interaural line (B). The location of the NCL is indicated between brackets, because this area was analyzed by (Nordquist et al., 2013), and here compared with our results and discussed. At 33 weeks of age, chicken brains were sampled from both the left (shown here) and right hemisphere (not shown); the grey dotted shapes illustrate the cutting lines per brain area.
Abbreviations: A = anterior to the interaural line; MSt = medial striatum; Thal = Thalamus; Arco = Arcopallium; Hippo = hippocampus; (NCL = nidopallium caudolaterale).

Statistical analysis

SAS version 9.2 was used for statistical calculations (SAS, 1989). Monoamine and metabolite levels per brain area were tested with a mixed model that included the fixed effects of line (LML vs. CL) and day (test day 1 and 2). Pen nested within line was added as a random effect to the model, thus, effectively, pen (n=16) was used as experimental unit for testing line effects. Post-hoc least square means were used to detect pair-wise differences. A log transformation for DA turnover in the arcopallium and HVA in the dorsal thalamus was executed to obtain normality of residuals. Data are presented as mean ± SEM. Effects of line on the behavioral responses of hens during the manual restraint test were analyzed using Kruskal Wallis tests as data were not normally distributed. If significantly different, values are presented as median (M) with the interquartile range, i.e. lower (Q1) and upper (Q3) quartiles. Many birds did not struggle or vocalize at all during manual restraint. Therefore, struggling and vocalizing during the test was also analyzed as a binary (yes/no) variable using a generalized mixed model with logit link function. Line and day were fixed effects in this model, and pen nested within line was added as random effect. Data are presented as mean ± SEM.

RESULTS

Effects on DA

In **Fig. 3.2**, the levels of DA, its three metabolites DOPAC, HVA, and 3-MT, NA and the calculated DA turnover in the arcopallium are shown. DA levels ($F_{(1,14)}$ = 4.1, P = 0.06) and levels of its metabolite HVA ($F_{(1,14)}$ = 4.1, P = 0.06) tended to be lower for LML birds than for CL birds. LML birds also showed significant lower NA levels ($F_{(1,14)}$ = 5.6, P = 0.03) and DOPAC levels ($F_{(1,14)}$ =7.1, P = 0.02) in this brain area compared to CL birds. Levels of 3-MT ($F_{(1,14)}$ = 0.0, P = 0.95) and the DA turnover ($F_{(1,14)}$ = 1.0, P = 0.24) in the arcopallium were unaffected by line. **Table 3.1** shows the levels of DA, DOPAC, HVA, 3-MT, DA turnover, and NA in the dorsal thalamus, medial striatum, and hippocampus per line. DOPAC levels in the thalamus were lower for LML birds compared to CL hens ($F_{(1,14)}$ = 6.1, P = 0.03). LML hens showed a higher DA turnover in the hippocampus ($F_{(1,14)}$ = 5.2, P = 0.04) than CL hens. No other line effects were found for DA and its metabolites in the dorsal thalamus, medial striatum, and hippocampus.

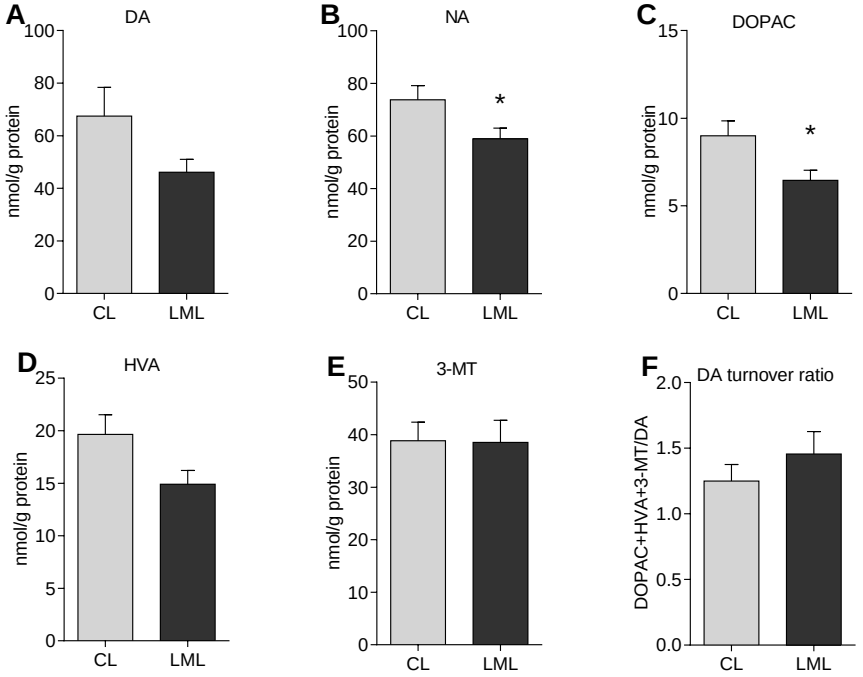

Fig. 3.2 Catecholamine and metabolite levels, and DA-turnover ratio in the arcopallium of adult laying hens. Mean (± SEM) values for catecholamines dopamine (A) and noradrenaline (B), the DA-metabolites DOPAC (C), HVA (D), and 3-MT (E), and the DA turnover ratio ((DOPAC+HVA+3-MT)/DA) (F) in the arcopallium of hens from the control line (CL) and low morality line (LML). n = 20/group; *P<0.05

Table 3.1 Catecholamine and metabolite levels, and DA-turnover ratio (mean ± SEM) in the dorsal thalamus, medial striatum, and hippocampus of hens from the control line (CL) and low morality line (LML)

	Control (CL)	Low mortality (LML)	*P*-value
Dorsal thalamus			
DA	12.33 ± 1.64	8.72 ± 0.98	0.105
NA	94.90 ± 6.71	81.77 ± 4.23	0.145
DOPAC	0.94 ± 0.13	0.55 ± 0.07	**0.027 ***
HVA	3.91 ± 0.65	2.89 ± 0.25	0.200
3-MT	6.79 ± 0.65	6.73 ± 0.56	0.944
DA turnover	1.05 ± 0.09	1.18 ± 0.08	0.219
Medial striatum			
DA	748.75 ± 34.11	701.90 ± 59.08	0.536
NA	38.25 ± 4.28	31.20 ± 3.17	0.211
DOPAC	33.70 ± 2.20	30.34 ± 2.89	0.421
HVA	45.60 ± 3.03	43.00 ± 4.01	0.726
3-MT	86.10 ± 5.93	95.55 ± 8.28	0.369
DA-turnover	0.23 ± 0.01	0.24 ± 0.01	0.291
Hippocampus			
DA	4.40 ± 1.09	2.95 ± 0.67	0.320
NA	66.95 ± 3.70	65.20 ± 3.01	0.723
DOPAC	0.95 ± 0.26	1.15 ± 0.26	0.600
HVA	1.35 ± 0.40	2.45 ± 0.59	0.145
3-MT	18.05 ± 1.91	19.25 ± 2.45	0.774
DA turnover	0.65 ± 0.15	1.52 ± 0.39	**0.043 ***

Statistically significant results are indicated as * (P<0.05); in nmol /g protein; n = 20/ group

Table 3.2 Serotonin and metabolite levels, and 5-HT-turnover ratio (mean ± SEM) in the dorsal thalamus, medial striatum, and hippocampus of hens from the control line (CL) and low morality line (LML)

	Control (CL)	Low mortality (LML)	*P*-value
Dorsal thalamus			
5-HT	92.84 ± 5.56	98.96 ± 5.33	0.533
5-HIAA	10.16 ± 0.65	10.01 ± 0.51	0.919
5-HT turnover	0.11 ± 0.01	0.10 ± 0.00	0.246
Medial striatum			
5-HT	159.60 ± 10.23	162.30 ± 10.88	0.850
5-HIAA	15.45 ± 0.97	14.75 ± 1.26	0.733
5-HT turnover	0.1 ± 0.00	0.09 ± 0.00	0.161
Hippocampus			
5-HT	177.25 ± 11.00	174.00 ± 10.13	0.853
5-HIAA	23.65 ± 1.15	23.85 ± 1.29	0.904
5-HT turnover	0.14 ± 0.01	0.14 ± 0.00	0.982

Mean± SEM in nmol / g protein; n = 20/ group

Effects on 5-HT

Fig. 3.3 shows levels of 5-HT, 5-HIAA, and 5-HT turnover in the arcopallium. There, 5-HIAA levels tended to be lower for LML hens compared to CL hens ($F_{(1,14)}$ = 3.6, P = 0.08). Levels of 5-HT ($F_{(1,14)}$ = 2.3, P = 0.15) and the 5-HT turnover ($F_{(1,14)}$ = 0.6, P = 0.45) in this area were unaffected by line. In the three other brain areas, i.e. the dorsal thalamus, medial striatum, and hippocampus, no effects of line on 5-HT, 5-HIAA, or 5-HT turnover were found (**Table 3.2**).

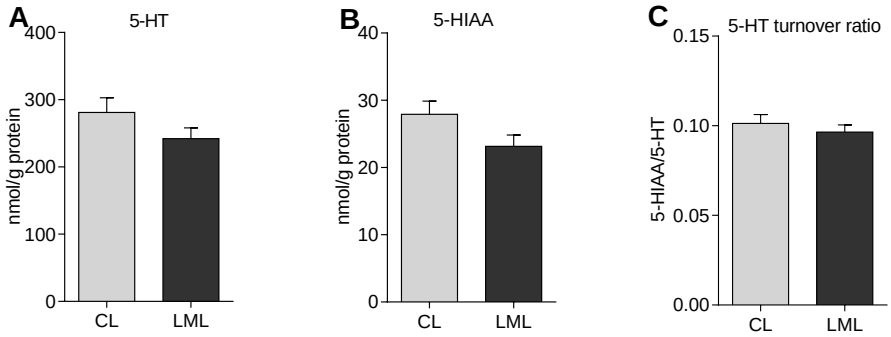

Fig. 3.3 Serotonin and metabolite levels and 5-HT-turnover ratio in the arcopallium of adult laying hens. Mean (± SEM) values for serotonin (5-HT) (A), its metabolite 5-HIAA (B), and the 5-HT turnover ratio (5-HIAA/5-HT) (C) in the arcopallium of hens from the control line (CL) and low morality line (LML), n = 20/group

Manual restraint

During the 5-min manual restraint, birds from the two lines differed in their latency to vocalize (χ^2 = 5.9, df = 1, P = 0.02) and frequency (χ^2 = 6.1, df = 1, P = 0.01), with LML birds vocalizing sooner (M_{LML} = 296 sec, $Q1$ = 161, $Q3$ = 300) and more (M_{LML} = 0.5, $Q1$ = 0.0, $Q3$ = 16.3) compared to CL birds (M_{CL} = 300 sec, $Q1$ = 300, $Q3$ = 300, and, M_{CL} = 0.0, $Q1$ = 0.0, $Q3$ = 0.0, respectively). When vocalizing was expressed as a binary variable, 50% of all LML hens vocalized, whereas only 15 % of CL hens did ($F_{(1,14)}$ = 3.60, P = 0.08). Lines did not differ significantly in their latency to struggle (χ^2 = 1.9, df = 1, P = 0.167; M_{LML} = 300, $Q1$ = 241, $Q3$ = 300 vs. M_{CL} = 300, $Q1$ = 300, $Q3$ = 300) or in frequency of struggling (χ^2 = 2.6; df = 1; P = 0.11; M_{LML} = 0, $Q1$ = 0, $Q3$ = 1 vs. M_{CL} = 0, $Q1$ = 0, $Q3$ = 0). Also, the percentage of birds that struggled during the test did not differ between LML (45%) and CL birds (20%) ($F_{(1,14)}$ = 2.31, P = 0.15).

DISCUSSION

The current study compared brain monoamine levels in four different brain regions of laying hens selected for low mortality using group selection of siblings (LML) with a control line (CL). Selection for low mortality resulted in changes in dopaminergic measures, most prominently present in the arcopallium, but did not significantly affect serotoninergic measures.

Effects of selection for low mortality on dopaminergic measures

Selection for low mortality resulted in lower levels of NA and DOPAC and a tendency for lower levels of DA and HVA, with no line differences for 3-MT levels and DA turnover ratio in the arcopallium. In agreement with this observed difference in dopaminergic action, a recent immunohistochemistry study in the same selection lines showed lowered tyrosine hydroxylase concentrations in the nidopallium caudolaterale (NCL) in hens of the LML (second generation) as compared to the CL (Nordquist et al., 2013). Tyrosine hydroxylase catalyzes the production of DA from tyrosine (Daubner et al., 2011) and fewer enzymes might lead to a reduced synthesis of DA and diminished production of metabolites, as shown here. It is speculated that other mechanisms may be involved too, such as an altered activity of dopamine β hydroxylase and monoamine oxidase (MAO) that might affect monoaminergic neurotransmission (Eisenhofer et al., 2004). While Nordquist and collaborators (2013) focused on the NCL (Nordquist et al., 2013); we focused on the arcopallium, medial striatum, hippocampus, and the thalamus. The telencephalic areas arcopallium and medial striatum contain a higher distribution of dopaminergic fibers and D1 receptors than the NCL (Durstewitz et al., 1999b; Stewart et al., 1996). A microdialysis study in pigeons showed a high release of DA and high production of DA-metabolites in the striatal area; with a relatively low HVA/DOPAC ratio reflecting a fast reuptake by the dopamine transporter (DAT) (Bast et al., 2002). In the present study, lines did not differ in their dopaminergic levels in the MSt. As the hippocampus has very little expression of the DAT (Borgkvist et al., 2012), reuptake of released DA into the presynaptic terminal is hampered, resulting in low levels of DOPAC and HVA. This effect might be stronger in CL hens, which had a lower DA turnover ratio in this area than LML hens. Similar to the arcopallium, DOPAC levels in the thalamus were lower for CL hens than for LML hens.

As LML are selected for low mortality due to FP and cannibalism, these results confirm previously found relationships between FP and the dopaminergic system (Kjaer et al., 2004; Uitdehaag et al., 2011). It is shown that LML hens had a lowered DA metabolism compared to CL hens, most prominently seen in the arcopallium. It remains unknown, however, how much DA is released by the presynaptic cell thereby contributing to the levels of DOPAC (via reuptake of DA), 3-MT (via released DA) and HVA (via forming of DOPAC and 3-MT) (Eisenhofer et al., 2004). A microdialysis

study could provide more details on the functional aspects of monoamines as this technique enables measuring presynaptic release of DA (and 5-HT) and its metabolites (Bast et al., 2002).

Effects of selection for low mortality on serotonergic measures

Selection for low mortality tended to lower 5-HIAA levels in the arcopallium of LML hens, but 5-HT levels, albeit numerically lower in LML hens, were unaffected by line. No significant serotonergic effects were found in any of the three other brain areas either. Previously, it has been shown that LML hens had higher peripheral whole blood 5-HT levels (Rodenburg et al., 2009; Bolhuis et al., 2009) and a lower platelet 5-HT uptake (Rodenburg et al., 2009; Bolhuis et al., 2009) than CL hens. In addition, it was shown that hens displaying severe FP had increased 5-HT turnover in the dorsal thalamus and higher levels of the 5-HT metabolite in the medial striatum compared to non-peckers and victims of FP (Kops et al., 2013b). Similar results were found when comparing 5-HT turnover between a flighty, FP-prone line and a more docile, low FP line (Uitdehaag et al., 2011). This suggests that FP may be influenced by brain 5-HT, whereas selection for low mortality is probably affected by more or different traits, obscuring the link with central 5-HT.

Effect of dopaminergic changes in the arcopallium on behavior and motor control

The catecholamines, such as DA and NA, are known to play a role in motivational and reward-related motor and higher cognitive functions like impulsivity (Kalenscher et al., 2006). Pharmacological studies in both birds and mammals support the involvement of the central dopaminergic system in dysfunctional behaviors. For instance, increasing DA activity by administering a DA_2 receptor agonist induced stereotypies in pigeons (Cheng and Long, 1974), increased aggressive pecking in normally low aggressive chickens (Dennis and Cheng, 2011), and increased impulsivity in rats (Winstanley et al., 2010). Administering a DA_2 receptor antagonist, thus decreasing DA activity, reduced FP ratios in laying hens (Kjaer et al., 2004), while a DA_1 receptor antagonist decreased the behavior of already high aggressive chickens (Dennis and Cheng, 2011) and high impulsive rats (Winstanley et al., 2010), but not in low aggressive chickens or low impulsive rats. Thus, high dopaminergic levels in CL might (at least partly) lie at the basis of FP or impulsivity. Also, a large number of studies have demonstrated that fearfulness is related to FP behavior (Hughes and Duncan, 1972; Vestergaard et al., 1993; Jensen et al., 2005; Rodenburg et al., 2004; Bolhuis et al., 2009; Uitdehaag et al., 2008b; Rodenburg et al., 2005; Uitdehaag et al., 2008a). It is assumed that more fearful animals are more prone to display defensive aggression and are more likely to perform severe FP (Rodenburg and Koene, 2003; Rodenburg et al., 2004) and cannibalism (Keeling and Jensen, 1995; Lesch, 2005; Blokhuis and Beuving, 1993). In addition, within a group both fearfulness and FP may

be transmitted among pen-mates (de Haas et al., 2012; Uitdehaag et al., 2008b; Zeltner et al., 2000), and also victims of FP show increased fearfulness (Hughes and Duncan, 1972; Vestergaard et al., 1993) with possible activation of the ascending DA system, as seen in rodents suffering from repeated aggressive attacks (Barik et al., 2013). Here, the more active vocal behavior of LML hens during the manual restraint indicates lower fearfulness compared to CL. That is, a more active behavior during fear tests represents lower fear levels or a higher social reinstatement motivation in comparison to non-vocalizing and non-struggling behavior (Forkman et al., 2007) as seen in CL hens. As described before LML showed reduced cannibalistic toe pecking (Rodenburg et al., 2009) and are characterized as less fearful in numerous behavioral tests compared to the CL (Rodenburg et al., 2009; de Haas et al., 2012; Bolhuis et al., 2009; Nordquist et al., 2011), including the one in this study. The differences in the dopaminergic activity between LML (low DA activity) and CL (high DA activity) might thus underlie the intergroup differences in damaging behaviors.

Importantly, the strongest dopaminergic effects were found in the arcopallium. Before the avian neuroanatomical nomenclature was changed, the area in the most ventrolateral and posterior part of the bird telencephalon was called archistriatum. Based on a large amount of neurobiological evidence, the archistriatum is now subdivided into the somatomotor arcopallium and a cluster of subnuclei that constitutes the amygdala (Reiner et al., 2004; Cheng et al., 1999; Yamamoto et al., 2005). Lesions in the amygdala of Japanese quail increase fear behavior in an open field test, while arcopallium lesions decrease anxiety (Saint-Dizier et al., 2009), which fits with our results. It is also possible, though, that the quails' fearful state was mediated by motor output deficits as fearful quails remained longer immobile in the open field (Saint-Dizier et al., 2009). In addition, humans suffering from either obsessive-compulsive disorder or trichotillomania, a hair-pulling disease with similarities to FP (van Zeeland et al., 2009), both have impaired inhibition of motor response (Chamberlain et al., 2006). L-DOPA (L-3,4-dihydroxyphenylalanine) is known to improve motor learning and motor memory by increasing DA levels (Flöel et al., 2005; Pearson-Fuhrhop et al., 2012). Increased DA levels can, however, also induce stereotypies (Deviche, 1983) and deficits in the control of posture and motor activity (Nistico and Stephenson, 1979). Dopaminergic terminals are abundant in the arcopallium (Wynne and Güntürkün, 1995) and activate primarily D1-receptors (Durstewitz et al., 1998), thereby possibly increasing spike densities of pre-activated neurons (Durstewitz et al., 1999b; Durstewitz et al., 1999a). Consequently, the higher DA activity might lead to a more active motor output system in CL hens, with impulsivity or even hyperactivity (Kjaer, 2009) as underlying problems associated with FP and cannibalistic behavior. Vice versa, it is hypothesized that lower dopaminergic neurotransmission in LML hens decreases the probability that motor-processes related to FP and cannibalism are activated and are executed.

CONCLUSION

The present study shows that selection for low mortality using a sibling group selection scheme affects the dopaminergic neurotransmission of laying hens with possible implications for the motor output of FP and cannibalistic behavior, as suggested by the strong results found in the arcopallium, a somatomotor area. The stronger effect of line in the arcopallium compared to the absence of significant effects for dopamine or serotonin in the limbic MSt suggests that deficits in motor functioning might be at the base of these behaviors, although fear and even impulsivity might also affect FP and cannibalism. Future studies are needed (e.g. *in vivo* microdialysis) on the role of dopamine in FP and cannibalism to further investigate the underlying neural mechanisms.

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Chapter 4

Central monoamine levels and behavior in young and adult laying hens from two lines selected on high (HFP) and low (LFP) feather pecking

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SUBMITTED

ABSTRACT

Severe feather pecking (SFP) in laying hens is a detrimental behavior with possibly neurochemical deficits at the base of this problem. Recent neurological studies depicted conflicting results on the role of serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) during different developmental stages. To investigate the motivational background and the role of 5-HT and DA in SFP behavior, we studied behavior and central monoamine levels of hens from genetic lines divergently selected on High and Low Feather Pecking (HFP vs. LFP) at different ages. Interestingly, at 8 weeks of age, HFP had lower 5-HT and DA activity than LFP, whereas the pattern at 25 weeks of age was the other way around. Line differences were found in both emotion regulating and motor regulating areas. As expected from previous generations, HFP exceeded LFP in most types of allopecking, including SFP. In addition, HFP responded more actively in most behavioral tests conducted, and seem more impulsive or hyperactive in their way of coping with stress. This paper shows developmental trajectories of the neurochemical systems (5-HT and DA) inversed between high and low genetic selection lines, but whether this is a cause or consequence of SFP needs further investigation. Furthermore, SFP behavior seems closely related with a high locomotor activity which is in line with previous findings.

INTRODUCTION

Severe feather pecking (SFP) in laying hens is a detrimental behavior. It can be defined as the pecking at and pulling out of feathers of group mates, sometimes followed by feather eating (McKeegan and Savory, 1999). SFP can easily evolve to skin pecking and cannibalism resulting in mortality of recipients and is therefore a considerable welfare problem in laying hens on commercial poultry farms. SFP has multifactorial risk factors many of which are related to the physical and social environment of chickens and their internal (genetically influenced) state that determines how they will respond to the environment (Uitdehaag et al., 2011; Rodenburg et al., 2013; Gilani et al., 2013). For instance, external factors such as foraging materials, housing system, climate and light have been demonstrated to contribute to chickens' vulnerability to start SFP (Kjaer and Vestergaard, 1999; Johnsen et al., 1998; Gilani et al., 2013), but also the presence of fearful group members and an instable social group likely affect the sensitivity for SFP (de Haas et al., 2012). Internal characteristics of chickens prone to develop SFP have been suggested to have high fearfulness and/or anxiety levels (Rodenburg et al., 2009; de Haas et al., 2012; Hocking et al., 2001; Bolhuis et al., 2009; Nordquist et al., 2011), and a proactive coping style (Koolhaas et al., 1999; Korte et al., 1998). Recent studies on the behavior of chickens divergently selected on FP, reported that birds from the high feather pecking (HFP) line were more active compared to those of the low feather pecking (LFP) line, and possibly even hyperactive (Kjaer, 2009). Although either of these characteristics (fearfulness, proactive coping or hyperactivity) may predispose chickens to develop SFP, the underlying neural mechanisms for SFP are not clear.

It has been suggested that neurochemical deficits might lie at the base of the SFP problem. Recently, the role of brain monoamines serotonin (5-hydroytryptamine, 5-HT) and dopamine (DA) became of interest. Pharmaceutical studies have shown that haloperidol – a dopamine D2 receptor agonist known to increase dopamine release when acutely administered (Hernandez and Hoebel, 1989) – reduces SFP in adult chickens (Kjaer et al., 2004; Hernandez and Hoebel, 1989). Moreover, a chronic dietary supplementation of the 5-HT precursor tryptophan, leading to enhanced 5-HT neurotransmission, decreased feather pecking (FP) in young chickens (van Hierden et al., 2004), whereas the 5-HT_{1A} autoreceptor agonist S-15535, inhibiting 5-HT release, increased the incidence of FP in young chicks (van Hierden et al., 2005) and adult hens (Dennis et al., 2008a). Brain analyses pointed out that young chickens displaying high FP incidences had lowered serotonergic and dopaminergic turnover compared to chickens with low FP (van Hierden et al., 2002; van Hierden et al., 2004; Bordnick et al., 1994; Kjaer et al., 2004; van Hierden et al., 2005; Biscarini et al., 2010; Flisikowski et al., 2009). Recent brain analysis in adult hens provided seemingly conflicting results: phenotypically and genotypically selected adult high feather peckers had a higher 5-HT turnover (Uitdehaag et al., 2011; Kops et al., 2013b) and a higher DA metabolism (Kops et al., 2013a) than low feather peckers. Thus, relationships between FP tendencies and brain neurochemical levels seem to vary between ages, but also SFP

behaviour itself is not constantly present in chickens. Young chickens predominantly perform gentle feather pecking (GFP), whereas most SFP incidences are recorded when laying hens reach reproductive maturity (McAdie and Keeling, 2002; Uitdehaag et al., 2011; Rodenburg et al., 2013). Moreover, GFP in young chicks is not a predictor of SFP in adult hens and not all animals will perform SFP (Rodenburg et al., 2013; Rodenburg et al., 2004; Jones et al., 1995). Importantly, previous brain studies have been performed in lines not directly selected on SFP but on related traits such as productivity (van Hierden et al., 2002) or mortality due to SFP and cannibalism (Van Hierden et al., 2002; Kops et al., 2013a). Genetic selection on SFP behavior has resulted in divergent feather pecking lines, called the high feather pecking (HFP) and low feather pecking (LFP) lines (Kjaer and Sørensen, 1997). These lines do not only differ in SFP, but also in other behavioral and physiological characteristics such as general hyperactivity (Kjaer, 2009; de Haas et al., 2010; Rodenburg et al., 2010; Kjaer and Guemene, 2009). In order to more clearly study the role of 5-HT and DA in SFP, this study aimed to compare these monoamines in brain areas involved in both emotional regulation and motor control between HFP and LFP hens, both when young and early into lay. This was combined with behavioral tests and observations to learn more on the animals' behavioral characteristics. During observations, the prevalence of gentle, severe, aggressive and toe pecking was recorded.

MATERIAL AND METHODS

Ethical statement

The experiment was approved by the Animal Care and Use Committee of Wageningen University, and in accordance with Dutch legislation on the treatment of experimental animals the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive. Animals were visually checked daily for signs of wounds as a consequence of SFP so that it was possible to react immediately when animal welfare was compromised.

Birds and housing

White leghorn hens from the 9th generation of divergently selected lines for high feather pecking (HFP) and low feather pecking (LFP) were used. Details regarding the selection procedure have been described previously (Su et al., 2005; Kjaer et al., 2001). Eggs of both HFP and LFP birds were brooded and after hatch, the one-day old female chicks received a neck tag with a color/number combination for identification. In total 84 female chicks were distributed over 12 pens (42 chicks/line; n=7/pen). Birds were not beak-trimmed. The chicks were housed in floor pens (1.9 by 1.2 m) covered with paper (first 7 weeks) or sawdust (after week 7). Water and a commercial mash diet were provided *ad libitum*: a starter diet (week 1-5), a grower diet (week 6-16) and a layer diet (from week 17 onwards). Each pen had a 50 cm high perch installed and a

lower perch (a block of wood) in the first seven weeks. Continuous light was given the first week, then 18 h of light (week 2) followed by 13 h (week 2 - 3), and 10 h of light (week 4 – 15). From 17 weeks of age onwards, the light period was extended by 1 h per week, until the birds had 16 h of light between 2.00 am – 6.00 pm at 23 weeks of age, in line with commercial practice. In the first two weeks, three chicks turned out to be male and four chicks had died. In week 8, each group was reduced by one chicken and the brains of these chickens were dissected and stored (n=6/line) (referred to as young). The group size was now 65 animals (n=32 LFP; n=33 HFP, n = 5 or 6 per pen). At an age of 10 weeks, the pullets were moved to a new animal facility. Group composition remained the same, but the pens were now in one row of 12 pens instead of two rows of 6 pens. In week 23, two hens per pen were selected for microdialysis, as described elsewhere (n=12/line) (Kops et al. submitted). In week 25, 25 animals (n = 12 LFP; n=13 HFP) were culled and the brains were dissected and stored (referred to as adult).

Behavioral observations and tests

Birds were individually subjected to six behavioral tests, which are described below. In addition, pecking behaviors were scored between 2 and 16 weeks of age. Order of testing and observations was always balanced for lines and pens. The experimenter was blind to the allocation of lines.

Pecking observations

Pecking behavior of each bird was weekly observed per pen from week 2 till week 16 with exception of week 9 and 10. Each observation lasted 25 min. At the start of each observation, the experimenter sat in front of the pen and waited for 5 min until starting with behavioral recordings. Frequencies of gentle feather pecking (GFP), severe feather pecking (SFP), toe pecking, and aggressive pecking were recorded at individual level. GFP was defined as light pecks given at the feathers; SFP was vigorous pecking and/or the pulling of feathers resulting in feather damage and/or removal; toe pecking was pecking directed at toes of others with risk of damaging the skin; and aggressive pecking consisted of forceful pecks at the head (Leonard et al. 1995). Pecking behaviors were averaged over weeks 2-8 for young birds, and weeks 10-16 for older birds, and expressed as frequencies per hour.

Isolation test

At an age of 8-9 days, each chick was subjected to an isolation test, carried out on two consecutive days. The chick was put in a round bucket (diameter 28 cm) outside the home pen, but in the same room as its home pen. For 2 min, the latency to move, the latency to vocalize, the number of vocalizations and attempts of escape were recorded.

Runway test

At an age of 15-16 days, each chick was subjected to a runway test, which was carried out on two consecutive days. The wooden runway of 160 cm (length) by 25 cm (height) by 20 cm (width) had a start box (20 by 20 cm) and a goal box (20 by 20 cm) at both ends of the runway closed with a steel mesh door. Three stimulus chickens of similar age and sex, which were not part of the experiment, were placed in the goal box. The tested chick was placed in the start box, and after 1 min, the mesh door was removed and the chick was given 5 min to get to its conspecifics. Latency to move, time to reach goal box, number and latency to vocalize and the number of defecations were recorded. The average time to conspecifics was calculated as (time to goal box) minus (latency to move). In the runway test, only 14.6% of the birds performed at least one escape attempt (fly). Therefore, escape was analyzed as a binary variable (yes/no).

Novel object test

At 23 days of age, the response to a novel object was tested. The novel object was a wooden block (5 by 5 by 2 cm) wrapped with colored tape (red, yellow, white, and green).The novel object was placed on the floor or bottom of the pen. The experimenter stood in front of the pen and recorded the latency of each bird to approach the object at a distance (radius) of 25 cm, 15 cm, or 10 cm. Birds that did not approach the object within the maximum test time of 5 min, were given the maximum time score. As many birds did not approach the object within 25 cm or closer during the test, approaching at 25 cm was also scored as a binary variable (yes/no).

Human approach test

At 31 days of age, a human approach test was conducted. One experimenter squatted in front of the opened door of the pen and stretched her arm inside the pen while the gaze was averted (head turned away) (Welfare Quality, 2009). Another observer stood aside and recorded the latency of each animal to approach the persons hand at a distance (radius) of 25 cm, 15 cm, or 10 cm. Birds that did not approach the person within the maximum test time of 5 min, were given the maximum time score. Also, the binary variable of approaching the person at 25 cm (yes/no) within the total observation time of 5 min was scored.

Open Field

Each bird was individually subjected to an open field (OF) test for 5 min at an age of 16 weeks (Rodenburg et al., 2009) for a detailed description of the test set-up). Birds were tested on two consecutive days. A square barren observation pen measuring 1.25 by 1.25 m (4.1 by 4.1 ft) operated as OF. Three walls and the flooring were wooden and one wall was from see-through perspex. The ceiling was covered with wire mesh.

Behavior was scored from a video-screen in an adjacent room by two persons using The Observer software package (Noldus Information Technology B.V., Wageningen, The Netherlands). Two cameras were placed, one in front of the see-through window and one above the OF. Durations and latencies to walk, stand, sit and vocalize (Collias, 1987) were recorded, as well as the number of vocalizations. Chicks were transported to and from the OF in a cardboard box.

Manual restraint

At 17 weeks of age, each hen was individually subjected to a manual restraint (MR) test, using a method previously described (Bolhuis et al., 2009). In short, a hen was placed on her right side on a table covered with cardboard, with the right hand of the experimenter covering the hen's trunk and the left hand gently stretching the hen's legs. Hens were retained in this position for 5 min. Consecutive struggles were scored as escape attempts, after which the hen was brought back in start position. The number, frequency and latency to vocalize were also recorded. All hens were tested at the same day by two observers and the test was situated in a room adjacent to their home pens.

Brain regions investigated and tissue preparation

Brain regions Seven regions of interest were selected because of their involvement in emotional and motor regulation (**Fig. 4.1**). The diencephalic dorsal thalamus connects with telencephalic areas (36) and disinhibition of the thalamus will affect goal-directed behavior (van den Heuvel et al., 2010). The telencephalic medial striatum (MSt) is together with the nucleus accumbens considered the limbic avian ventral striatum known to play a role in reward (Cai et al., 2011; Husband and Shimizu, 1999). The hippocampus is considered the memory and learning area in both mammals and birds (Colombo and Broadbent, 2000). Being a large associative area in the chickens' brain, the nidopallium has a potential role in guiding motor actions and decision making (Reiner et al., 2004; Güntürkün, 2005; Jarvis et al., 2013). The nidopallium is subdivided in the caudolateral nidopallium (NCL) with frontal-like executive functions (Güntürkün, 2012) and the limbic caudocentral nidopallium (NCC) (Shanahan et al., 2013; Atoji and Wild, 2009). Both the NCL and the NCC have reciprocal projections to the arcopallium intermedium, a somatosensory area, and the arcopallium mediale, a limbic region (Metzger et al., 1998; Kröner and Güntürkün, 1999; Atoji and Wild, 2009). Together with other subnuclei that surround the arcopallium intermedium, the arcopallium mediale is considered the birds' amygdala (Reiner et al., 2004; Cheng et al., 1999; Yamamoto et al., 2005). Therefore, in the paper 'arcopallium' refers to the arcopallium intermedium and 'amygdala' refers to the sampled arcopallium mediale.

Brain tissue preparation Brains of young (8 weeks of age) and adult hens (25 weeks of age) were removed and immediately deep frozen in n-heptane put on dry ice and stored at -80°C (protocol by van Hierden et al., 2002; Van Hierden et al., 2002). Slicing of brains was executed in a cryostat (Frigocut Jung Mod_700) under cold conditions (-10°C). Slice thickness was 400 µm. The seven regions of interest were located using the brain atlas for 2-week-old chickens by Puelles et al. (Puelles et al., 2007) with considering literature on the avian brain (Metzger et al., 1998; Atoji and Wild, 2009; Metzger et al., 2002), and also taking into account that brain size had increased in the 25 week old hens. **Fig. 4.1** is a schematic drawing depicting the location of the medial striatum (MSt), dorsal thalamus (thal), hippocampus (hipp), NCC, NCL, arcopallium (arco), and amygdala (amy). The gray dotted shapes illustrate the cutting lines per brain area (carefully cut with a scalpel). Tissue samples were taken from multiple slices ("A" is anterior to the interaural line and the abbreviations reflect those in the atlas (Puelles et al., 2007): Medial striatum (A7.56 - 5.68 mm, MSt) including the nucleus accumbens (A8.08 - 7.56 mm, Acb), dorsal thalamus (A2.80 - 1.36 mm; DPe, DMA, DIA, DLA), hippocampus (A4.24 – 1.60 mm; Hi1, Hi2, PHiM, PHil, PHil1, PHil 2, and PHiA), NCC (A3.04 – 1.36 mm; lateral parts of the caudal island of the nidopallium, NCIF), NCL (A3.04 – 2.56 mm), arcopallium (A3.28 - 2.08 mm; amygdala core regions 1-4), and the amygdala (A2.80 - 1.60 mm; amygdaloid Taenial nucleus; ATn). Brain samples of the left and right hemisphere were taken together and analyzed as one. Brain samples were weighted before analyzed.

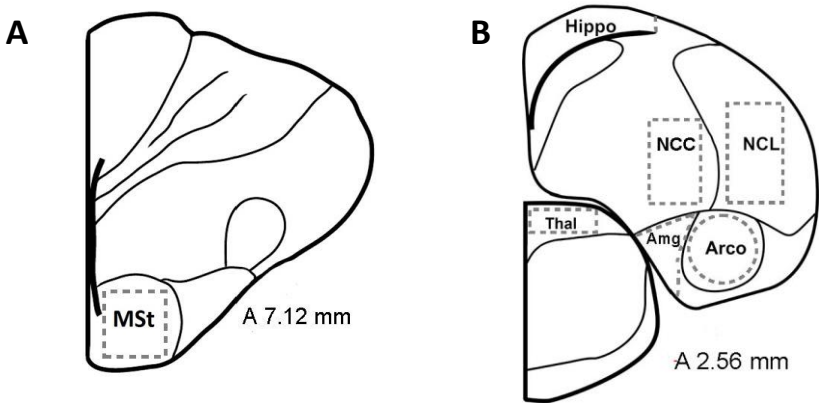

Fig. 4.1 Coronal schematic views of the chicken brain illustrating the medial striatum, thalamus, hippocampus, NCL, NCC, arcopallium, and amygdala. The schematic views of the left hemisphere of a chicken's brain. Depicted are the medial striatum (MSt) at 7.12 mm anterior to the interaural line (A), and the thalamus (thal), hippocampus (hipp), NCL, NCC, arcopallium (arco), and amygdala (amg) at 2.56 mm anterior to the interaural line (B). Chicken brains were sampled from both the left (shown here) and right hemisphere (not shown); the grey dotted shapes illustrate the cutting lines per brain area.

Determination of monoamines and metabolite content of brain tissue by HPLC

Concentrations of central serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and dopamine (DA) with corresponding metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and also noradrenaline (NA) and tryptohphan (TRP) in the brain tissue were measured by high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) after acid deproteinization as previously described (Kops et al., 2013a). Monoamine concentrations are expressed as nmol/g. Turnover ratios of serotonin (5-HIAA/5-HT) and dopamine ((DOPAC+HVA)/DA) were calculated as an index for the activity of the serotonergic and dopaminergic system (Korte-Bouws et al., 1996; Hallman and Jonsson, 1984); high levels indicate a higher monoamine neurotransmission.

Statistical analysis

SAS version 9.2 was used for statistical calculations (SAS, 1989). Data of behavioral tests were analyzed using mixed models containing the fixed effect of line (HFP vs. LFP) and the random effect of pen within line. In the case of the isolation test, runway test and open field test, also test day was added as a fixed effect. Person was added as a fixed effect for the manual restraint. Binary data were analyzed with a generalized mixed model with a logit link function. Brain measurements were analyzed with a mixed model containing the fixed effect of line (HFP vs. LFP). Prior to analysis, if needed, variables were logarithmically (durations in the behavioral tests and brain monoamine measurements) or square root (frequencies in behavioral tests) transformed to obtain normality of residuals. Data are presented as (untransformed) means ± SEM. The assumption of normality was invalid for frequencies of pecking behavior, even after transformation, and therefore the non-parametric Kruskal Wallis test was applied.

RESULTS

Pecking observations

At a young age, HFP chicks gave significantly more severe (χ= 5.0, P<0.05) and toe pecks (χ= 5.2, P<0.05) than LFP chicks. HFP and LFP chicks did not differ significantly in giving gentle pecks (χ = 0.65, P>0.10), see **Table 4.1**. Later, as adolescent, HFP still gave more severe pecks than LFP (χ= 12.63, P<0.01), but HFP also gave more gentle pecks (χ= 10.8, P<0.01) and now showed aggressive pecking behavior as well (χ= 8.6, P<0.01). Toe pecking decreased and did not differ between lines at this older age. Furthermore, also when expressed as proportion of chickens performing particular types of pecking, line differences were found (**Table 4.2**). Over

90% of all young chicks gave gentle pecks ($F_{(1,79)}$= 0.6, P>0.1) whereas more HFP (97.0%) than LFP (78.1%) tended to give gentle pecks when older ($F_{(1,63)}$= 4.0, P<0.1). At both ages, significantly more HFP birds showed severe pecking at least once (young: $F_{(1,79)}$=4.4, P <0.05; older: $F_{(1,63)}$= 9.7 , P<0.01). Proportion of birds displaying toe pecking was only significantly different at a young age (young: $F_{(1,79)}$ = 4.8,P <0.05; older: $F_{(1,63)}$=0.92, P>0.1). More HFP than LFP birds showed aggressive pecking, which was only observed at an older age (older: $F_{(1,63)}$=5.8, P<0.05) compared to LFP.

Table 4.1 Gentle, severe, toe, and aggressive pecking young chickens and adolescent hens of LFP and HFP line (mean ± SEM)

	Young			Adolescent		
Pecking behavior[1]	LFP (*n*=40)	HFP (*n*=41)	*P*-value	LFP (*n*=32)	HFP (*n*=33)	*P*-value
Gentle	4.77 ± 1.54	5.98 ± 1.42	*ns*	2.50 ± 0.53	7.20 ± 1.77	<**0.01**
Severe	0.41 ± 0.31	0.94 ± 0.40	**<0.05**	0.09 ± 0.05	0.98 ± 0.33	**<0.01**
Toe	0.26 ± 0.10	0.59 ± 0.14	**<0.05**	0.03 ± 0.03	0.09 ± 0.06	*ns*
Aggressive[2]	-	-		0.01 ± 0.01	0.17 ± 0.05	**<0.01**

[1]Average of number of pecks per bird per hour (age 2-8 weeks; 175 min total observation time per bird) when young and adolescent (age 11-16 weeks; 150 min total observation time per bird).
[2]Aggressive pecks only occurred during observations between weeks 13-16.
Data (untransformed) are represented as means ± SEM, *ns* is non-significant (*P*>0.10).

Table 4.2 Percentage of LFP and HFP chickens giving gentle, severe, toe and aggressive pecks at young and adolescent age

	Gentle			Severe		
%Give[1]	LFP	HFP	*P*-value	LFP	HFP	*P*-value
Young	95.0	90.2	*ns*	17.5	39.0	**<0.05**
Adolescence	87.1	97.0	**=0.05**	15.6	54.5	**<0.01**

	Toe			Aggressive[2]		
%Give[1]	LFP	HFP	*P*-value	LFP	HFP	*P*-value
Young	25.0	48.8	**<0.05**	-	-	
Adolescence	3.1	9.1	*ns*	3.1	30.3	<0.05

[1]Proportion of chickens (LFP or HFP) giving pecks at least once (yes/no) when young (age 2-8 weeks; 175 min total observation time; (LFP: *n* =40; HFP: *n* = 41) and older (age 11-16 weeks; 150 min total observation time;LFP: *n* =32; HFP: *n* = 33).
[2]Aggressive pecks were only recorded in weeks 13-16. *ns* is non-significant (*P*>0.10).
Statistically significant results are depicted in bold (*P*<0.05)

Behavioral tests

The results of the behavioral tests are shown in **Table 4.3**.

Isolation test
Chicks of the HFP line vocalized significantly sooner ($F_{(1,10)}$=5.0, P<0.05) and walked sooner ($F_{(1,10)}$=6.1, P<0.05) in the isolation test than chicks from the LFP line. Lines did not differ in the number of vocalizations in the isolation test.

Runway test
LFP and HFP did not differ in latency to vocalize or walk in the social runway test. The latency to join conspecifics after start of the test tended to be shorter in HFP than in LFP ($F_{(1,10)}$=3.9, P<0.10). When the latency to walk was subtracted from this measure (i.e. latency to conspecifics – latency to walk) the line difference was, however, not significant ($F_{(1,10)}$=2.7; LFP vs. HFP: 38.5±10.90 vs. 22.8±7.35 sec).

Novel object test
HFP chicks were faster in the 25 cm radius ($F_{(1,10)}$=5.3, P<0.05) than LFP, as a higher proportion of HFP chicks approached the novel object to a distance of 25 cm or closer compared to the LFP chicks ($F_{(1,10)}$= 5.33, P<0.05). HFP chicks were faster in the 25 cm radius ($F_{(1,10)}$=5.26, P<0.05).

Human approach test
Compared to LFP, HFP entered the 25 cm radius faster ($F_{(1,10)}$=11.0, P<0.01);
twice as much HFP chicks of an age of 31 days approached the human to a distance of 25 cm or closer compared to LFP chicks ($F_{(1,10)}$=11.5, P<0.01).

Open Field test
In the open field, lines did not differ in the number of steps or vocalizations, or in the latency to vocalize. The HFP hens yawned significantly more often than the LFP ($F_{(1,10)}$=5.1, P<0.05).

Manual restraint
In the manual restraint, chickens of the HFP line vocalized sooner ($F_{(1,10)}$=15.34, P<0.01) and more often ($F_{(1,10)}$=8.63, P<0.05) than the LFP. HFP struggled later ($F_{(1,10)}$=5.58, P<0.05) and less frequently ($F_{(1,10)}$=5.17, P<0.05) than the LFP.

Table 4.3 Isolation test, runway test, novel object test and human approach test in young chickens (8-31 days) and open field test and the manual restraint test in adolescent chickens (16-17 wks) of the HFP and LFP line.

Variables	LFP	HFP	*P*-value
Isolation test (8-9 days)			
Latency to voc (s)	25.3 ± 3.73	14.6 ± 3.53	<**0.05**
Number of vocs	37.5 ± 5.25	54.2 ± 8.19	ns
Latency to walk (s)	35.7 ± 5.93	12.7 ± 3.54	<**0.05**
Runway test (15-16 days)			
Latency to voc (s)	16.2 ± 4.65	19.6 ±7.95	*ns*
Number of vocs	54.1 ± 5.15	56.1 ± 4.31	ns
Escape attempts (% of birds)	20.0	9.5	ns
Latency to walk (s)	23.6 ± 10.75	13.2 ± 6.83	*ns*
Latency to conspecifics (s)	62.2 ± 14.76	36.0 ± 12.09	<0.10
Novel object test (23 days)			
Approach object (% birds)	52.5	83.3	**<0.05**
Lat. to 25 cm radius (s)	178.5 ± 19.48	118.4 ± 18.13	<**0.05**
Human approach test (31 days)			
Approach human (% of birds)	45.0	90.5	<**0.01**
Lat. to 25 cm radius (s)	202.4 ± 18.59	60.3 ± 14.39	<**0.01**
Open Field (16 weeks)			
Number of steps	31.3 ± 5.02	33.5 ± 5.76	ns
Number of vocalizations	66.5 ± 12.63	61.8 ± 10.33	*ns*
Latency to vocalize (s)	94.5 ± 15.57	80.8 ± 15.81	*ns*
Yawning	0.4 ± 0.35	2.6 ± 0.92	<**0.05**
Manual restraint (17 weeks)			
Latency to vocalize (s)	144.6 ± 18.37	71.2 ± 14.96	<**0.01**
Frequency of vocalizations	23.2 ± 3.69	57.2 ± 9.19	**<0.05**
Latency to struggle (s)	173.3 ± 17.62	230.3 ± 16.60	<**0.05**
Number of struggles	2.0 ± 0.36	1.2 ± 0.35	<0.10

Age is in between brackets. Data (untransformed) are represented as mean ± SEM, ns is non-significant; significant differences between groups are indicated by <0.05 or <0.01; a trend by <0.10.

Serotonergic line differences in young and adult chickens

In general, young LFP had higher serotonergic levels compared to young HFP (8 weeks of age), while these differences appeared less prominent in adult chickens (25 weeks of age) (**Fig. 4.2**, see pg. 64). Five of the seven brain areas in young chickens depicted significant line differences for either 5-HT, its metabolite (5-HIAA), or the turnover ratio (5-HIAA/5-HT). That is, young LFP had higher 5-HT ($F_{(1,10)}$=9.1, P<0.05) and higher 5-HIAA in the dorsal thalamus ($F_{(1,10)}$=8.1, P<0.05), the medial striatum ($F_{(1,10)}$= 7.5, P<0.05), the amygdala ($F_{(1.10)}$=15.7, P<0.05), and the NCC (F $F_{(1.10)}$=12.9, P<0.01). Also, compared to young HFP, young LFP had a higher 5-HT turnover ratio in the medial striatum ($F_{(1.10)}$=7.3, P<0.05), the NCC ($F_{(1,10)}$=8.2, P<0.05), and in the arcopallium ($F_{(1,10)}$=5.1, P<0.05). Moreover, TRP levels significantly differed between the young lines in the NCC ($F_{(1,10)}$=12.9, P<0.05) and the NCL ($F_{(1.10)}$=16.3, P<0.05) with young LFP (NCC: 30.5±1.34 ; NCL 33.6±1.13) having higher levels than young HFP (NCC: 23.3±1.52; NCL 33.6±1.13).

In contrast, in 25-week old chickens almost all significant serotonergic effects in similar brain areas had disappeared; except for 5-HIAA in the arcopallium ($F_{(1,21)}$=4.4, P<0.05) and 5-HT turnover ratio in the NCC ($F_{(1,21)}$=4.7, P<0.05). Opposite to the results at a young age, adult HFP had higher 5-HT turnover ratios compared to adult LFP.

Dopaminergic line differences in young and adult chickens

Dopaminergic line differences were found in both young and adult chickens (**Fig. 4.3**, see pg. 65). Young 8-week old LFP chicks had a higher DA turnover in both the dorsal thalamus ($F_{(1.10)}$=7.6, P<0.05) and medial striatum ($F_{(1.10)}$=8.0, P<0.05) compared to young HFP. In the 25-week old chickens these particular areas did not depict line differences, whereas the arcopallium and NCC did. That is, adult LFP had higher DA ($F_{(1.21)}$=10.6, P<0.01) and higher DOPAC ($F_{(1.21)}$=4..5, P<0.05) compared to adult HFP in the NCC. In contrast with results at a younger age, DA turnover in this area was significantly higher in adult HFP than in adult LFP ($F_{(1.21)}$=5.2, P<0.05). HFP had also significantly higher levels for DOPAC in the arcopallium (F(1.21)=6.7, P<0.05). No line differences in NA were found in any of the brain areas (not shown), except in the NCL. There, young LFP had significantly lower NA compared to HFP ($F_{(1.10)}$=6.3, P<0.05; LFP vs. HFP: 0.17±0.12 vs. 0.24±0.13), whereas LFP had higher NA than HFP when adult ($F_{(1.21)}$=6.3, P<0.05; LFP vs. HFP: 0.23±0.04 vs. 0.08±0.04).

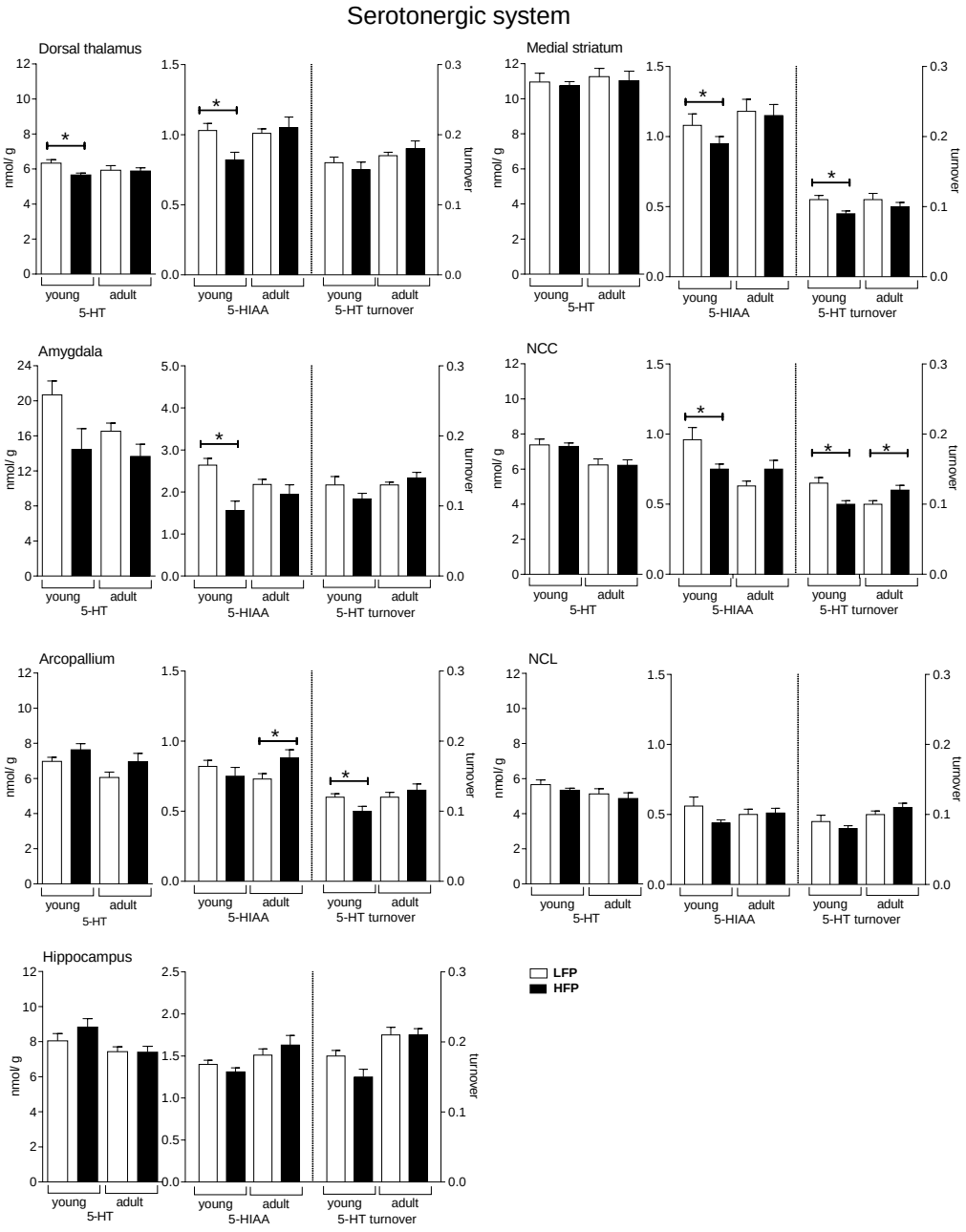

Fig. 4.2 Serotonergic concentrations in seven brain areas of young (8-week old) chickens (LFP: *n*=6, HFP: *n*=6) and adult (25-week old) chickens (LFP: *n*=12, HFP: *n*=13). For each brain area, the two most left panels show concentrations of 5-HT and 5-HIAA in nmol/g and the right panel shows turnover ratio. Statistically significant results are indicated as * ($P<0.05$). White bars are LFP, black bars are HFP.

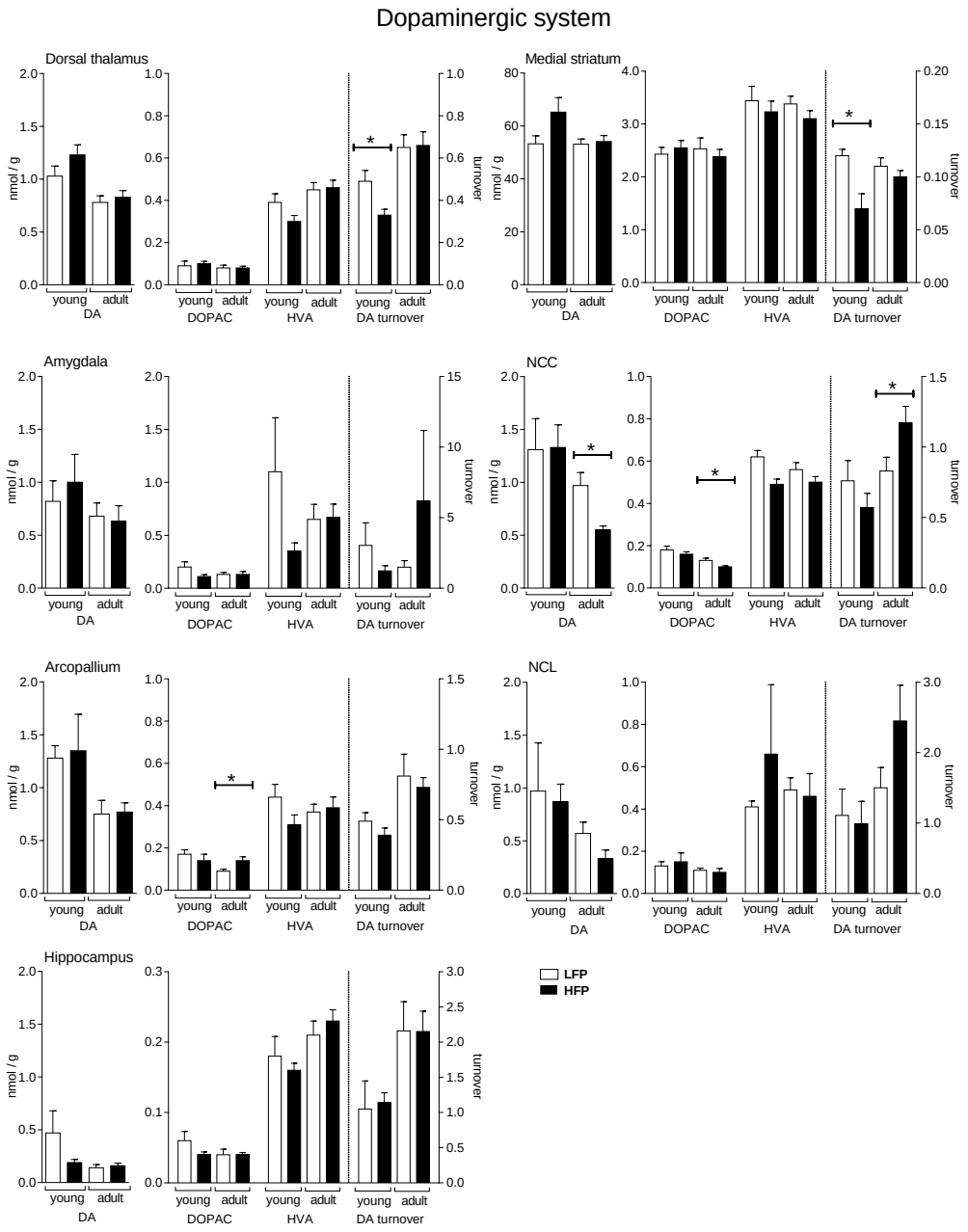

Fig. 4.3 Dopaminergic concentrations in seven brain areas of young (8-week old) chickens (LFP: *n*=6, HFP: *n*=6) and adult (25-week old) chickens (LFP: *n*=12, HFP: *n*=13). For each brain area, the two most left panels show concentrations of DA and metabolites DOPAC and HVA in nmol/g and the right panel shows turnover ratio. Statistically significant results are indicated as * ($P<0.05$). White bars are LFP, black bars are HFP.

DISCUSSION

Pecking in young and adolescent chickens

Behavioral observations in this study demonstrate line differences in all the types of allopecking investigated (GFP, SFP, toe- and aggressive pecking) even though the LFP and HFP lines have been selected only on their level of severe feather pecking (SFP) (Kjaer et al., 2001). Only GFP at a young age was, at a similar frequency, observed in almost all birds in both lines which can be explained by the social explorative character of that type of pecking (Riedstra and Groothuis, 2002). Line differences in allopecking behavior seen already at a young age in the present study were similar to those observed in previous studies with these lines (Bessei et al., 2013; de Haas et al., 2010; Rodenburg et al., 2010; Harlander Matauschek et al., 2010). Also, a higher proportion of HFP chicks pecked others compared to LFP chicks, which might explain the higher levels of allopecking in the HFP line. Still, this suggests that divergent selection on severe feather pecking induces an effect on general pecking activity.

Behavioral tests comparing lines

The behavioral responses of the birds in the different tests conducted may reflect different underlying traits and motivations, including fearfulness (reviews by Forkman et al., 2007; Jones, 1996). In several studies, HFP has been related to high fearfulness and anxiety (Hughes and Duncan, 1972; Vestergaard et al., 1993; Jensen et al., 2005; Rodenburg et al., 2004; Bolhuis et al., 2009; Uitdehaag et al., 2008b; Rodenburg et al., 2005; Uitdehaag et al., 2008a). The isolation test and the runway test examine a birds' fear of social isolation and the sooner or more chicks vocalize or walk (to others), the more motivated they are to join their conspecifics and to avoid detection by predators (Gallup Jr. and Suarez, 1980). HFP birds had a shorter latency to vocalize and move when isolated and they tended to move faster to their conspecifics in the runway. This active behavior may suggest that HFP are more fearful than LFP. Conflicting with these results, however, is the lack of line differences in activity in the open field test, a validated measure of fearfulness in chickens (Forkman et al., 2007). A tonic immobility test conducted in similar lines also revealed no differences in fear levels within these lines (Rodenburg et al., 2010). Moreover, almost all HFP were fast in approaching the novel object/person compared to only half of the LFP approaching, which would indicate lower fearfulness in HFP in this specific test situation. Strikingly, all these tests show that HFP respond faster and more actively than the LFP. Kjaer (2009) showed a higher general locomotor activity in HFP chickens than in LFP ones, which was not a consequence of increased feather pecking (performing or receiving); rather it seemed a trait-specific result of selection for SFP behavior. In line with this, we suggest differences in the way the birds cope with challenging conditions, i.e. a more active coping style, (hyper)activity and/or impulsivity, rather than fearfulness,

might underlie the fast and active responses of the HFP observed in our behavioral tests. Previously, it has been suggested by Koolhaas and co-workers (2007) that behavioral coping styles and emotionality can be seen as two independent dimensions of stable trait characteristics.

The serotonergic and dopaminergic system in young and adult LFP and HFP hens

In this study, 5-HT and DA (and their metabolites) concentrations were measured in seven brain areas of chickens originating from the HFP and LFP selection lines (Kjaer and Sørensen, 1997). At 8 weeks of age, young LFP had higher serotonergic activity (mostly higher 5-HIAA concentrations or higher 5-HT turnover ratio) compared to young HFP in all brain areas except the NCL and hippocampus. Also, LFP had higher DA turnover in the dorsal thalamus and the medial striatum when young. This is in agreement with previous literature on 5-HT and DA activity in the rostral forebrain of young chickens differing in FP (van Hierden et al., 2002). At 25 weeks of age, lines differed less prominent, but still serotonergic and dopaminergic differences were found in the NCC and the arcopallium. Strikingly, now the HFP had higher DA and 5-HT turnover in the NCC as compared to the LFP. This fits with our previous findings in adult birds with a propensity to perform SFP (Kops et al., 2013b; Kops et al., 2013a).

Monoaminergic systems in *young* chickens divergently selected on severe feather pecking

The current study demonstrates that young LFP and HFP lines predominantly differ in serotonergic activity, and to a lesser extent, dopaminergic activity in the dorsal thalamus, medial striatum, amygdala, NCC and arcopallium. These brain areas are involved in guiding motor actions and decision-making by integrating sensorimotor, motivational and emotional information (Reiner et al., 2004; Güntürkün, 2005; Jarvis et al., 2013; Lewis and Todd, 2007). Furthermore, studies in birds have shown these brain areas are innervated by both serotonergic and dopaminergic fibers (Herold et al., 2011a; Metzger et al., 2002; Stewart et al., 1996; Durstewitz et al., 1998). Lesions in, for instance, the limbic medial striatum impaired the suppression of immediate reward seeking behavior in chicks (Izawa et al., 2003) – thus made chickens more impulsive – and lesions in the limbic amygdala (to recall, the amygdala sampled here refers to the arcopallium mediale) increased fear in quails (Saint-Dizier et al., 2009). The arcopallium seems also involved in anxiety, although lesions in the arcopallium (intermediate part) decreased fear in chicks (Izawa et al., 2003). Moreover, the arcopallium is a somatosensory area involved in motor output (Reiner et al., 2004; Cheng et al., 1999; Yamamoto et al., 2005). Mostly strong serotonergic differences were found in these 8-week-old chicks. At that age, the brain is still in development, and this phase of brain maturation, and is characterized by high neuroplasticity. This lasts until chicks are 10 weeks of age (Rogers, 1995; Atkinson et al., 2008). This shaping of brain networks is coordinated by 5-HT, but 5-HT is also essential for

synapse formation during adulthood (Lesch and Waider, 2012; Rostas et al., 1991). Abnormal concentrations of 5-HT can have a long-lasting effect on brain circuitries and with that, on the behavioral output (Lesch and Waider, 2012). For instance, mice lacking brain 5-HT (by a mutation on the tryptophan hydroxylase 2 (Tph2) gene responsible for the 5-HT producing enzymes) have abnormalities of the serotonergic circuitry formation in several rostral brain areas; increasing the risk to develop anxiety (Migliarini et al., 2012). Recently, it was shown that 5-HT administration in the embryonic phase and also in the post-hatch period altered aggressiveness and fearful behavior later in life, up to adolescence (Dennis et al., 2013b; Dennis et al., 2013a). In addition, genetic factors, stress and other environmental influences such as social isolation, in general affect neuroplasticity (Patzke et al., 2009; Cheng and Fahey, 2009) and modulate monoaminergic pathways in the chick's forebrain (Gruss and Braun, 1997). Overwhelming evidence from human studies shows a role for 5-HT and early deficits in frontal-striatal-thalamic brain areas in neurological/psychiatric disorders such as schizophrenia, anxiety disorders, impulse control disorders, compulsive disorders, and attention deficit/hyperactivity disorders (ADHD) (van den Heuvel et al., 2010; Chamberlain et al., 2009; Fineberg et al., 2010; Insel et al., 1985; Pattij and Vanderschuren, 2008; Bechara et al., 1994; Chamberlain and Sahakian, 2007). Interestingly, compared to typically developing children, children with ADHD have a delay in cortical, prefrontal, maturation (Shaw et al., 2007). The hyperactive and impulsive component of ADHD behavior seems under the control of 5-HT also mediating dopaminergic functioning (Quist et al., 2001). 5-HT is known to directly affect locomotor activity as well (Jacobs et al., 2002; Gerson and Baldessarini, 1980). Therefore, it is speculated that the general higher behavioral activity of HFP birds in the different tests might be related to their low activity of the serotonergic system and corresponding affected neuronal circuits.

Monoaminergic systems in *adult* chickens divergently selected on severe feather pecking

At the age of 25 weeks, line differences in adult birds were restricted to the NCC and the arcopallium, and in the opposite direction as observed in young birds. HFP laying hens had both higher 5-HT and DA turnover ratios in the NCC and higher DOPAC and 5-HIAA levels in the arcopallium compared to LFP. This latter area also showed similar DA-metabolite differences in lines selected on low mortality as a consequence of SFP and cannibalism (Kops et al., 2013a). Remarkably, the NCC and arcopallium are strongly connected (Reiner et al., 2004; Güntürkün, 2005; Jarvis et al., 2013) and whereas the NCC predominantly displays limbic connectivity (Shanahan et al., 2013; Atoji and Wild, 2009), the arcopallium processes somatosensory information (Herold et al., 2011a; Durstewitz et al., 1998; Durstewitz et al., 1999a; Herold et al., 2012b). Therefore deficits in the monoaminergic system in these brain areas have the potential to affect both the emotional perception and behavioral output. Indeed, increased DA levels can induce stereotypies (Deviche, 1983) and deficits in the control of posture

and motor activity (Nistico and Stephenson, 1979). Similar to the arcopallium (Wynne and Güntürkün, 1995), dopaminergic terminals are abundant in the NCC and activate primarily D1-receptors (Durstewitz et al., 1998), thereby possibly increasing spike densities of pre-activated neurons (Durstewitz et al., 1999b; Durstewitz et al., 1999a). In our study, the higher DA turnover in the NCC of HFP compared with LFP hens, although DA concentrations were not different, shows that HFP have a higher metabolic DA activity. Previously, it was shown that chickens with a higher SFP-propensity showed a higher increase in locomotor response after acute administration of apomorphine, a DA-D_1 and D_2 receptor agonist, than birds with the lower propensity to SFP - reflecting a more sensitive dopaminergic system in the former (van Hierden et al., 2005). Consequently, the higher DA activity might lead to a more active motor output system in HFP, underlying hyperactivity. It is suggested that these higher levels in adult hens are responsible for more persistent pecking behavior. Yawning during the open field test was significantly increased in HFP in comparison to LFP birds and this comfort-like behavior might be a marker of recovery from acute stress (Dourish and Cooper, 1990). Yawning is under the control of the serotonergic and dopaminergic system, as increased activity of these systems – as seen in the HFP – has a facilitating effect on yawning (Argiolas and Melis, 1998).

In support of previous literature in different young and adult chickens (van Hierden et al., 2002; Kops et al., 2013b; Kops et al., 2013a), this paper shows a clear inversion of neurochemical markers over developmental time: where young high feather pecking hens are characterized by a low serotonergic and dopaminergic activity, adult HFP hens have a higher activity of these monoaminergic systems. For LFP the opposite accounts, the monoaminergic activity is first higher in young than lower in the adults. The fast, perhaps even impulsive reactions of HFP in various tests, fits in the behavioural profile of individuals with a hyperactivity disorder. It is suggested here that this enhanced locomotor activity is caused by the lower serotonergic activity affecting neural circuits at a young age. Possibly these birds activate their (impaired) serotonergic system by increasing locomotor activity thereby increasing 5-HT activity as seen later in life (Jacobs et al., 2002; Jacobs and Fornal, 1997), but more research is needed on the causation of this neurochemical shift during development and how hyperactivity can be related with SFP via the serotonergic and dopaminergic systems

CONCLUSION

This paper shows that divergent genetic selection on SFP has changed the serotonergic and dopaminergic system with possible implications for the underlying motivation for displaying SFP. Similar to previous studies in young chickens, low monoamine turnover ratios are related to HFP behavior; suggesting that the brain of HFP chicks is prone for certain behavioral deficits, such as the general behavioral (hyper)activity they display. We hypothesize that selection for severe feather pecking behavior has indirectly resulted in changes of predominantly the serotonergic system early in life,

with long-term behavioral and neurological consequences later in life Remarkably, differences between the low and high feather pecking lines in activity of neurochemical systems (5-HT and DA) observed at a young age were reversed at an adult age. Whether this is a cause or consequence of the behavioral differences between the lines - SFP and (hyper)activity - needs further investigation. Furthermore, SFP behavior seems closely related with a high locomotor activity which is in line with previous findings. More research is needed to further specify the role of the developing monoaminergic system in SFP and the accompanying hyperactivity and vice versa.

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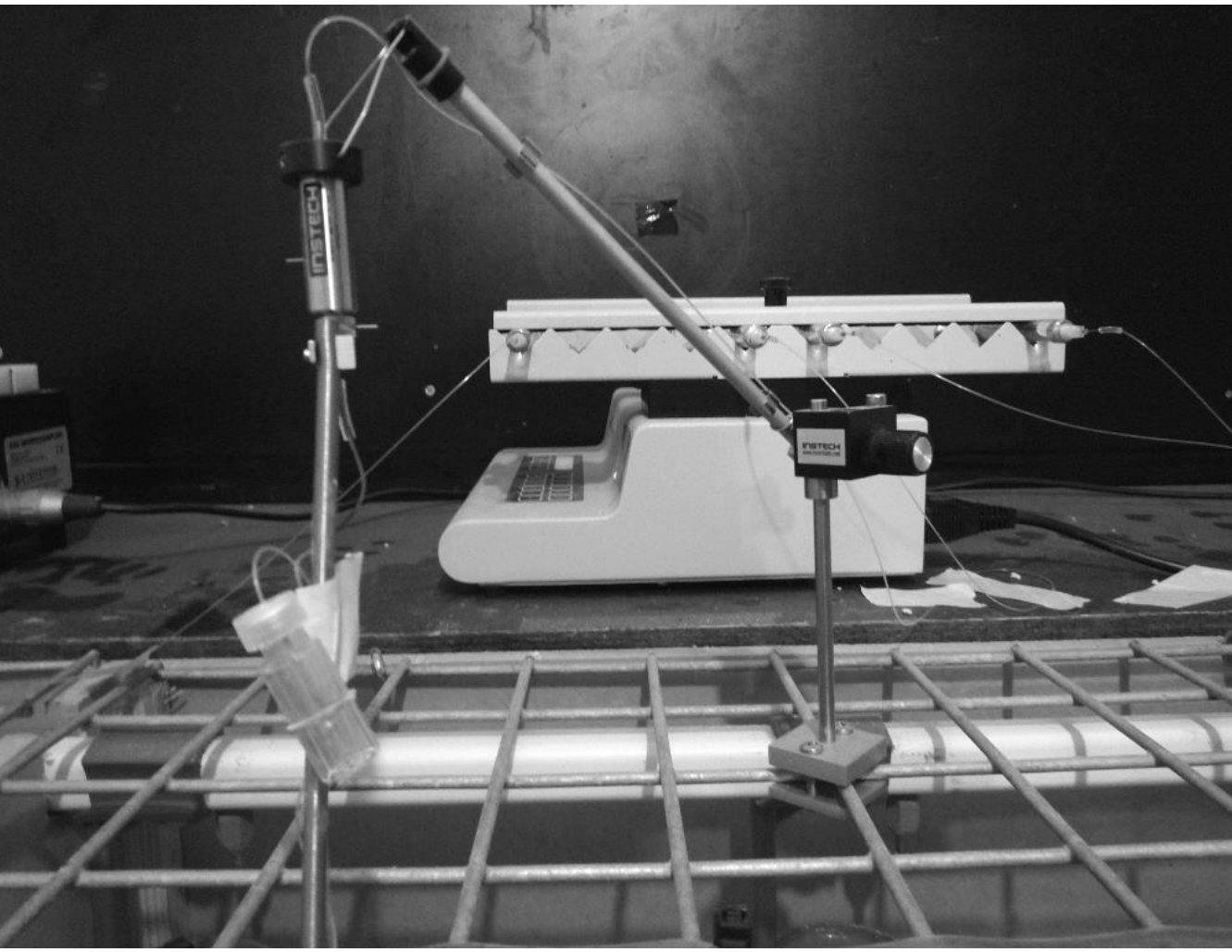
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Chapter 5

Successful brain microdialysis in freely moving adult chickens (*Gallus gallus domesticus*)

Marjolein S. Kops, Koen G.C. Westphal, Yvonne R.A. van Zeeland, Berend Olivier, J.Elizabeth Bolhuis, S.Mechiel Korte

SUBMITTED

ABSTRACT

Background *In vivo* brain microdialysis is a minimally invasive sampling technique that enables multiple measurements of brain monoamines in freely moving animals. This technique is predominantly used in small animals such as mice, rats, and small birds. Also young chickens (not older than 25 days) have been subjected to this technique, but literature shows no records of microdialysis in larger adult chickens.

New Method Recently, we successfully performed microdialysis in adult chickens. Here, we give a detailed description of the novel standardized method used, including important adjustments made to the stereotaxic apparatus, habituation to single housing, anesthetics, and probe installation and protection. In addition, vital parameters were recorded during surgery.

Results This novel approach has led to successful surgeries for microdialysis probe placements in all 24 adult chickens. Monoamines and metabolites were detected in samples of all but three adult chickens, with serotonin, its metabolite and dopamine metabolites clearly visible in the chromatograms.

Comparison with Existing Method In the past, microdialysis has been successfully performed in small birds, measuring brain monoamines. Here a new method is presented that is successful in measuring monoamines by microdialysis in larger adult birds.

Conclusions Microdialysis in freely moving adult chickens is feasible with the here presented protocol. This is particularly useful for the investigation of the role of monoamines in certain behaviors that are more prevalent in adults like detrimental feather pecking behavior. A remaining difficulty is the absence of a brain atlas with coordinates of the adult chicken brain.

MICRODIALYSIS IN CHICKENS

In vivo microdialysis is a minimally-invasive sampling technique that can be used for continuous measuring of chemical compounds in the extracellular fluid of both peripheral and central tissues while, at the same time, the tested animals are minimally disturbed (see reviews by (Westerink, 1995; Strüder and Weicker, 2001). When used for brain studies, this technique allows for measuring e.g. the extracellular release of brain monoamines (Westerink et al., 1987). Pioneers in the microdialysis technique are Bito (Bito et al., 1966), Delgado (Delgado et al., 1972) and Ungerstedt (Ungerstedt et al., 1982) and their colleagues in the '60s and '70s. In 2001, over 3000 articles referring to the microdialysis technique were published (Shippenberg and Thompson, 2001). Nowadays, a PubMed search yields over 10.700 articles on brain microdialysis of which over 8.300 studies performed in rats. Brain microdialysis in birds is much rarer: 35 articles refer to avian brain microdialysis with chickens (Gruss et al., 1999; Alam et al., 2012a; Matsunami et al., 2012), songbirds (Hamaguchi and Mooney, 2012), pigeons (Karakuyu et al., 2007; Herold et al., 2012a; Bast et al., 2002) and Japanese quail (Kleitz-Nelson et al., 2010) as birds of choice. The 19 studies on microdialysis in chickens are presented in **Table 5.1**. The main focus of these studies involves brain mechanisms of imprinting and feeding. The procedure in general comprises of a single probe approach, with or without additional use of a 'guide cannula'. The latter approach allows for multiple measurements over several days, while the former is in general used for a single day due to inherent restrictions of the probe. Strikingly, the age of the chickens subjected to microdialysis ranges from 1 to 25 days. No records were found on brain microdialysis in adult chickens, although several research groups have studied brain monoamines in chickens. In these research groups, a target region is selected and punched from histological slides of the brain of hens (van Hierden et al., 2002; Cheng and Fahey, 2009). This 'punch'-method, however, has the disadvantage that behavior of the animal and the target neurotransmitters cannot be studied simultaneously (as the brain is collected after the animal is sacrificed). In addition, the samples reflect a combination of both the monoamine concentrations in presynaptic storage vesicles and in the extracellular space, rather than providing separate information about the two. As a result, the proportion of neurotransmitters which resembles the concentration released remains unknown using this 'punch' method. Moreover, where this later method only provides a single measurement at a single time point, microdialysis offers multiple measurements at different time points. It allows for measuring the extracellular release of brain monoamines (Westerink, 1995); both at baseline and in response to for instance,

Table 5.1. Overview of the current in vivo brain microdialysis studies performed in young chickens

Reference	Animal	Age*	Method	Sex	Brain area	Dialysate and treatment	Anesthetic protocol
Alam et al., 2012a	Boris Brown layer	17-18	Guide cannula	male	VMH	DA after L-DOPA	Sodium pentobarbital (3 mg/100 bw), i.v
Alam et al., 2012b	Gallus G. domesticus, breed unknown	17-18	Guide cannula	male	VMH	DA, NE, 5-HT after oral lysine	Sodium pentobarbital (3 mg/100 bw), i.v
Zachar et al., 2012	Hunnia-broiler	1	Probe	both	Striatum	Aspartate and Glu after high potassium or distress	Ketamine and xylazine i.m
Matsunami et al., 2012	White Leghorn layer	7 to 14	Guide cannula	both	MST/ NAcc	5-HT and DA after fluvoxamine	Ketamine and xylazine i.m
Wagner et al., 2011	Hunnia Broiler	1	Probe	both	MST/ NAcc	Aspartate and Glu detected with fluorescent tags	Ketamine and xylazine i.m
Alam et al., 2011	Layer chicks, breed unknown	17-18	Guide cannula	both	VMH	DA after L-DOPA and lysine-free diet	Sodium pentobarbital (4 mg/100g bw), i.v.
Khalil et al., 2010	Broiler	18	Guide cannula	male	VMH	5-HT after cannula implantation	Sodium pentobarbital (4 mg/100g bw), i.v.
Hamasu et al., 2009	"Julia" layer	1 to 2	Guide cannula	male	MNH	DA, NE, 5-HT after l-proline	Sodium pentobarbital (3 mg/100g bw), route unknown
Ichijo et al., 2008	Layer chicks	17-18	Guide cannula	male	VMH	DA after lysine devoid diet	Sodium pentobarbital (3 mg/100g bw), i.p
Baldauf et al., 2005	Brown leghorn	38-44 g	Probe	both	MNH	5-HIAA,HVA, Glu and taurine after opioids (imprinting)	Halothane
Gruss et al., 2003	White Leghorn	1 (36-41 g)	Probe	both	MNH	DA (HVA), 5-HT (5-HIAA), Glu, taurine after haloperidol (imprinting)	Halothane
Tachibana et al., 2001	Gallus G. domesticus	17-18	Guide cannula	both	Medial and lateral hypothalamus	5-HT and NE (MHPG) after feeding	Sodium pentobarbital (3 mg / 100 g bw), i.v

Table 5.1. continued

Reference	Animal	Age*	Method	Sex	Brain area	Dialysate and treatment	Anesthetic protocol
Tachibana et al., 2000	Gallus G. domesticus	17-18	Guide cannula	both	Medial hypothalamus	NE (MHPG) after feeding	Sodium pentobarbital.(3 mg / 100 g bw), i.v.
Tsukada et al., 1999	White Leghorn	3	Guide cannula	both	MVH	ACh and Glu (imprinting)	Ether inhalation
Gruss et al., 1999	White Leghorn	2 (39-42 g)	Probe	both	MNH	Glu, taurine, 5-HIAA, HVA, (MHPG) after NMDA-receptor mediated modulation	Equithesin (0.3 mL/100 g body weight), s.c.
Daisley et al., 1998	White Leghorn	1	Probe	both	IMHV	Glu, aspartate, taurine, glutamine, glycine after passive avoidance training	Halothane inhalation
Gruss and Braun, 1996	White Leghorn	2 (39-42 g)	Probe	both	MNH	Glu after auditory filial imprinting and stress	Equithesin (0.3 mL/100 g body weight), s.c.
Gruss and Braun, 1996b	White Leghorn	2 (39-42 g)	Probe	both	MNH	Glu after auditory filial imprinting	Equithesin (0.3 mL/100 g body weight), s.c.
Hasegawa et al., 1994	Gallus G. domesticus	Unknown**	Probe	both	Pineal gland	Melatonin	Unknown

*age in days unless stated otherwise; **age was not specified in this study, but most pineal gland research is aimed at neonatal development of the pineal gland and studied in embryonic and early post hatch chicks via both *in vitro* and *in vivo* techniques (Karaganis et al., 2008; Zeman and Herichová, 2011; Robertson et al., 1990), thus most likely this microdialysis study is performed in chicks not older than 25 days.

Abbreviations brain areas (in alphabetical order): IMHV= intermediate medial hyperstriatum ventrale (Intermediate medial mesopallium, IMM, see Reiner et al., 2004); MNH = medio-rostral neostriatum / ventral hyperstriatum; MST/NAcc = medial striatum/nucleus accumbens; MVH = medial hyperstriatum ventrale; VMH = ventromedial hypothalamus

Abbreviations dialysate and treatment (in alphabetical order): 5-HT = 5-hydroxytryptamine (serotonin); 5-HIAA = 5-hydoxyindole-3-acetic acid; ACh = acetylcholine; DA = dopamine; Glu = glutamate; HVA = homovanillic acid; L-DOPA = L-3,4-dihydroxyphenylalanine; MHPG = 4-hydroxy-3-methoxyphenylglycol; NE = norepinephrine; NMDA = N-methyl-D-aspartate

antidepressants (Prins et al., 2011a). Measuring the release is of biological relevance as only after being released monoamines can excite pre- and postsynaptic receptors corresponding with adequate stimulation of the second messengers system or target organs (Strüder and Weicker, 2001). *In vivo* brain microdialysis is therefore, very applicable for studying the neurochemistry of animal behavior (Westerink, 1995; Benveniste and Hüttemeier, 1990).

Recently, the first successful microdialysis study in adult chickens was performed (Kops et al. 2013, submitted; **Chapter 6**). While using the technique in rats as template (Prins et al., 2011a; van Heesch et al., 2013; van der Stelt et al., 2005), some crucial adjustments needed to be made to be able to perform the procedure in adult chickens. One of the challenges encountered was that adult hens have, other than young chicks, a large comb on the head and base of the upper mandible. This comb is exactly at the level of where the nose bar of the adaptor rests on the upper mandible, thereby complicating fixing of the head for surgery. Another challenge was that the microdialysis infusion system contained very fragile, movable parts that had to stay intact while still allowing the awake, large adult chicken to make all desired head-movements. In addition, protective measures were taken for the probe itself as head-scratching with sharp toenails and vigorous head-shakes of chickens could be a real hazard to the functionality of the probe.

In this present paper, a detailed protocol is described that was used to successfully perform microdialysis in large adult chickens, including how to solve the issues raised above, the habituation to single housing, the surgery with gas anesthetics, the probe installation, the recovery from surgery and the details regarding the test day are discussed. Finally, we discuss the advantages and limitations of this technique when used in adult laying hens.

MATERIALS AND METHODS

Animals

Twenty-four 23-week-old White Leghorns hens (*Gallus gallus domesticus)* were used originating from the ninth generation of lines selected on high and low feather pecking (Su et al., 2005; Kjaer et al., 2001). Prior to the microdialysis procedure, hens were group-housed in pens (7 birds per pen). Each pen measured 1.9 by 1.2 m and floors were covered with paper (first 7 weeks) or saw dust (after week 7). Water and a commercial mash diet were provided *ad libitum*. For further details, see (Kops et al., 2013a). At week 23, 24 animals were randomly selected for microdialysis and habituated to individual housing and the test cages. Average body weight was 1.2 ± 0.3 kg (n=24). Throughout the experiment, animals were visually checked daily for signs of distress, discomfort, and wounds so that it was possible to react immediately when animal welfare was compromised. All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University, the Netherlands, and

found to be in accordance with Dutch legislation on the treatment of experimental animals, the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

Experimental housing and habituation prior to surgery

A novel environment and/or solitary housing may evoke a stress response in animals (Koolhaas et al., 1999; Gallup Jr. and Suarez, 1980; Suarez and Gallup Jr., 1985; Bolhuis et al., 2005). Solitary housing was considered essential for performing microdialysis because otherwise there would be the risk of tangled tubing and a risk of potential damage inflicted to the sensitive equipment by the other bird pecking at the probe. Chickens were therefore individually housed in a room adjacent to the operating room for the duration of the experiment. Housing consisted of wooden cages (l x w x h = 60 x 30 x 40 cm) with steel-wire mesh on top (see **Fig. 5.1**) and a Perspex window was installed in the front of the cage and in the U-shaped cut-outs in the partitions to still allow visual and vocal contact. The floor was covered with saw dust and a small perch (wooden block, l x w x h = 30 x 5 x 5 cm). Birds were allowed to acclimatize and habituate to this novel environment for 3 to 5 days prior to surgery. This habituation period furthermore allowed habituation to the presence of humans as the researchers walked regularly by the cages that were placed on top of tables, thereby lowering the fear response of chickens evoked upon confrontation with the researchers (Hocking et al., 2001). The cages used for microdialysis were of similar design as the habituation cages but did not contain side-windows to allow for undisturbed individual microdialysis measurements and had been customized such that the front window could open to function as a worktable; a slot was cut out for the microdialysis tubing to fit through. Light was controlled by a time switch (light period was 16h, 2.00 am – 6.00 pm). A radio was turned on during light hours. Temperature in the habituation room was on average 18.7±1.4^{o}C with an average humidity of 60.4±9.2%. Food and water was provided *ad libitum,* although food was withheld the morning of surgery. Chickens were put in a clean cage on the morning of habituation and cages were cleaned before animals returned from surgery.Microdialysis surgery

General check-up

Three surgeries were performed per day, with microdialysis executed the subsequent day. Because of the bird's size, two researchers prepared the hen for surgery, particularly with respect to the intubation, the fixation of the head in the stereotaxic instrument and combining the surgery with the monitoring of the vital parameters during the surgical procedure. On the morning of surgery, food was withheld three hours prior to surgery (Curro, 1998). The birds received a health check and were weighed. Water remained available at all times.

Stereotaxic apparatus and surgical microscope

A rat stereotaxic apparatus (Model 902, David Kopf instruments, Tujunga, California, USA) was made equipped for chickens by raising it 15.2 cm (6 inch) and installing a special Chicken/Duck adaptor (model 917, David Kopf instruments) (**Fig. 5.2a**). During surgery, the position of the adaptor and the head was checked with a custom-made ruler (**Fig. 5.2b**). The 45° adaptor slide (Model 1246 45 Degree Adaptor Slide, David Kopf instruments, Tujunga, California, USA) was adjusted to an angle of 43° following the stereotaxic brain atlas by (Puelles et al., 2007) (**Fig. 5.2c**). This atlas deviates from the atlas by (Kuenzel and Masson, 1988) requiring an angle of 45°. The stereotaxic instrument was used to determine the three-dimensional coordinates of the probe. The anterior/posterior (AP) and medial/lateral (ML) coordinates were determined by the interaural line, this is the midpoint of the ear bars. The dorso/ventral (DV) coordinates was later determined from the dura (see section Probe installation). A surgical microscope (model 6370, Zeiss, Sliedrecht, the Netherlands) was used for the precise measurements. Importantly, a pilot study was performed to establish the precise coordinates of the brain region of interest as the adult brain of chickens does not precisely follows the coordinates described by the stereotaxic atlas by Puelles et al. (2007) as this is based on two-week-old chicks.

Preparation of the microdialysis probe

Prior to the surgery, a microbiotech MAB 4.11.2 Cu microdialysis probe (Microbiotech, Stockholm, Sweden) had to be perfused for at least 10 min (to remove all glycerol present in the probe). To do so, the probe was clamped in the probe holder on the stereotaxic apparatus. PE Tubing (Instech Laboratories, Inc., Plymouth, Pennsylvania, USA) was attached with connection pieces (Gilson, Villiers-le-Bel, France) to both in- and outlet of the probe with an infusion pump linked to the inlet set on a 2 µl/min perfusion rate. Perfusion fluid was Milli Q (Millipore, Synergy UV, Billerica, Massachusetts, USA). After perfusion, the AP and ML coordinates were determined. The tubing was therefore removed and the tip of the probe was brought exactly in between the ear bars of the stereotaxic instrument.

Anesthetics and intubation

Sevoflurane (SevoFlo, Abbott Animal Health, Chicago, Illinois, USA) was used to induce a rapid and effective general anesthesia. This inhalant anesthetic provides a both a rapid induction and recovery, and does not induce profound cardiopulmonary depression, organ toxicity or wing flapping, unlike isoflurane (Curro, 1998; Lierz and Korbel, 2012; Joyner et al., 2008; Jaensch et al., 1999). The sevoflurane was delivered with an Ohmeda TEC 7 vaporizer (Datex-Ohmeda, Inc., Madison, Wisconsin, USA), via a Mapleson A semi-open system (Jackson-Rees, Medline Industries, Inc., US). Anesthesia was induced with 7-8 vol% sevoflurane in 100 % oxygen (O_2) delivered via

a face mask (see **Fig. 5.3a** for the anesthetic equipment). O_2 -flow was set on 0.65 l/min for the entire procedure. Once the bird was sufficiently anesthetized, as indicated by closure of the eyelids, loss of voluntary movements, and absence of a toe pinch withdrawal reflex, the bird was intubated using a Hudson RCI Sheridan cuffed endotracheal tube (Teleflex Medical Inc., Athlone, Ireland) with an internal diameter (ID) of 3.0 or 3.5 mm according to a technique described by (Curro, 1998). In short, to visualize the glottis of the chicken, the tongue is gently grasped with a forceps and pulled forward. By lightly pressing a fingertip gently against the throat the tongue will lift. The tube is again gently inserted directly into the trachea. Prior to insertion of the tube, the tip was lubricated with silicone spray (Silkospray, Willy Rüsh GmBH, Kernen-Rommelshausen, Germany) and securely taped to the lower beak using leukotape (BSN Medical, Almere, the Netherlands). Although a cuffed tube was used, the cuff was not inflated as this may pose a risk of necrosis and tracheal stenosis (Abou-Madi, 2001; Lennox, 2004). Anesthesia was maintained using 3-4 vol% sevoflurane. In contrast to rats, no ointment was applied to the eyes of the chickens as the birds already close their eyes when anesthetized (Lierz and Korbel, 2012) and the ophthalmic ointment may have detrimental effects to the bird's plumage.

Analgesia

Pre-operative analgesia used were carprofen (Rimadyl, 50 mg/mL, Pfizer Animal Health, Capelle aan de IJssel, the Netherlands; 5 mg/ kg BW SC) and butorphanol (Dolorex 10 mg/mL, Merck Animal Health, Schiphol-Rijk, the Netherlands; 1 mg/kg BW SC). After intubation, these analgesics were administered subcutaneously in the 5.1inguinal area. Local analgesia (xylocaine (2%) and adrenaline, Alfasan Nederland BV, Woerden, the Netherlands) was later used on the skull membranes.

Monitoring of vital parameters during surgery

Throughout the entire procedure, the bird's respiration rate (RR) was monitored via Ohmeda respiratory gas monitor (5250 RGM, Ohmeda, Louisville, Colorado, USA), see **Fig. 5.3b**. In addition, the O_2- levels and carbon dioxide (CO_2) levels in the exhaled air were monitored using a capnograph attached to this monitoring device. The pulse rate (PR), and O_2-saturation levels in the blood (SpO_2) were recorded using a Doppler flow device taped to the paw and attached to the Ohmeda monitor. Body temperature was measured both at the start and the end of the procedure using a thermometer (MT 1831, Microlife AG, Widnau, Switserland). The chicken was positioned in lateral recumbency on the right side in between the bars of the raised stereotaxic instrument on a thermostat-regulated heating mat set to intermediate heating level. It had to be noted that the Ohmeda anesthetic machine had settings installed to anesthetize pigs and therefore the cut-off point for the pulse rate was saved at 255 beats/min.

Fig. 5.1

Fig. 5.4

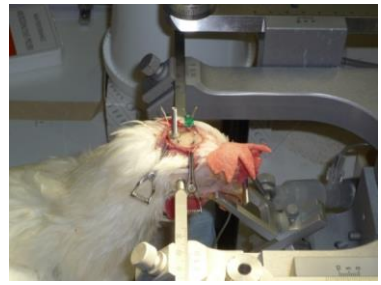
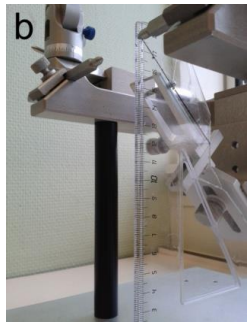


Fig. 5.2: a, b, c



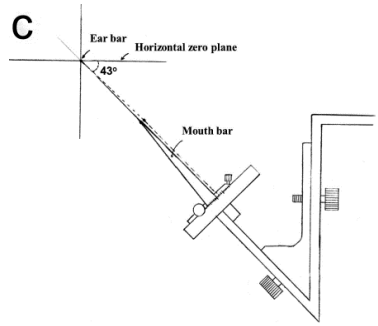


Fig 5.3: a, b

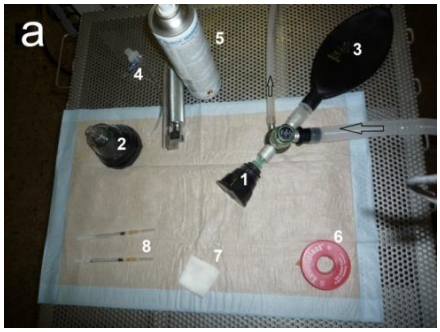


Fig 5.5: a, b

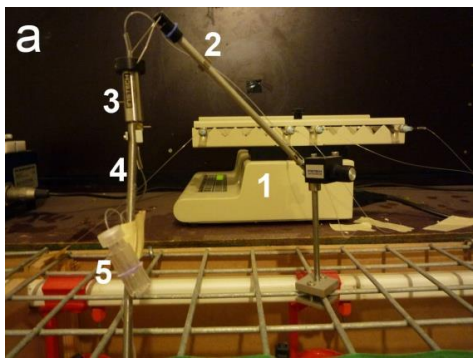


Fig 5.6: a - f

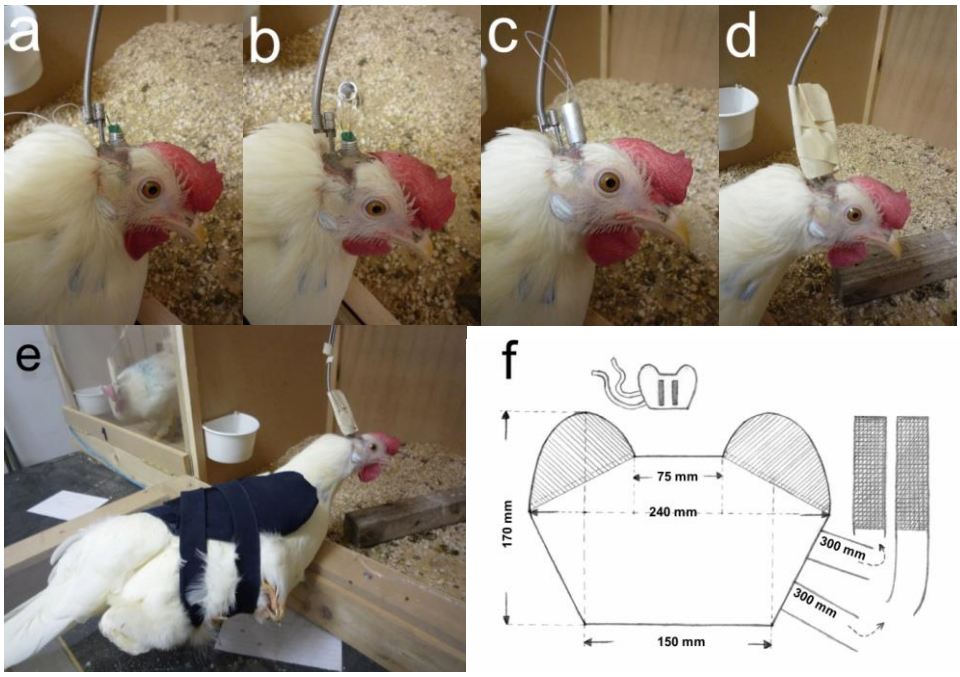


Fig 5.7: a, b

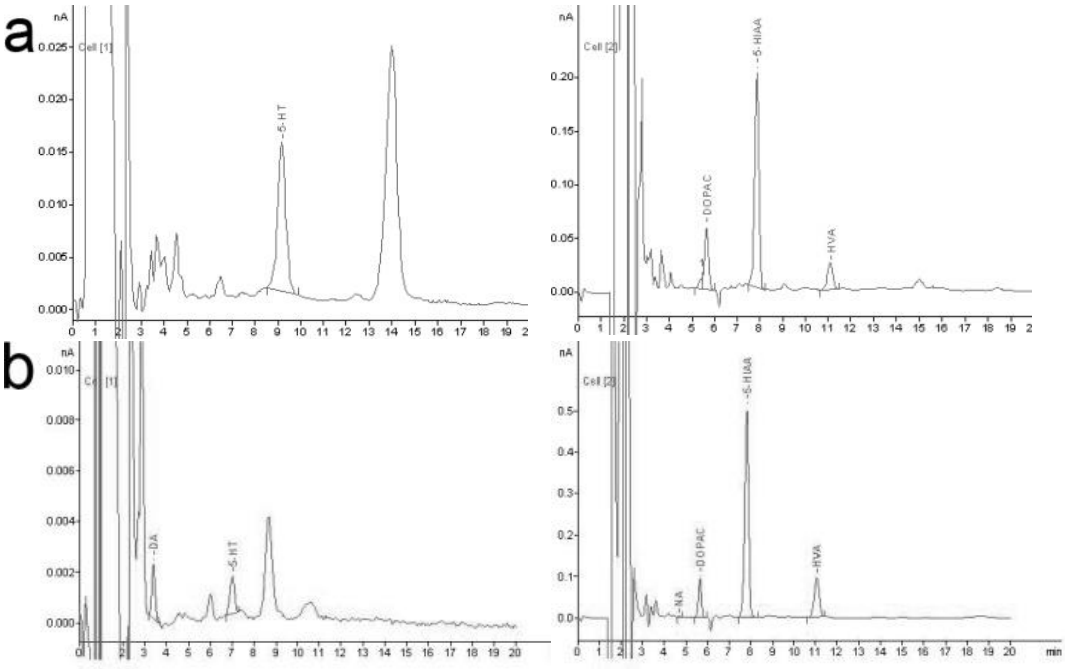


Fig. 5.1 Habituation cages (60 x 30 x 40 cm) with steel-wire mesh top and Perspex windows installed in the front of the cage and in the U-shaped cut-outs in the partitions. Saw dust covered the floor. White food cup and red drinking nipples provided food and water. Not on photo: small wooden perch (30 x 5 x 5 cm).

Fig. 5.2 Stereotaxic apparatus: (a) front view and (b) side view of the raised stereotaxic apparatus with customized ruler. (c) A schematic drawing of the Chicken/Duck adaptor illustrating how to adjust the mouth bar to the angle of 43^{o}, following (Puelles et al., 2007) (adaptor nose bar is not depicted).

Fig. 5.3 Equipment for anesthetizing and intubating chickens (a) and the Ohmeda gas monitor displaying vital parameters during surgery (b).(a) Anesthesia equipment: Mask (1) and spare mask (2) for inhalation induction; rebreathing bag (3); endotracheal tube (4); silicone spray to lubricate the tip of the tube (5); leukotape to tape the tube to the lower mandible (6); gauze with 70% alcohol to clean the inguinal area (7); injection needles with analgesia (8); not on photo: forceps to grab and pull the tongue out to ease intubation, and thermometer used for monitoring body temperature during the procedure. (b) Monitor displaying from left to right: CO_2–levels and backflow (in mmHG), respiration rate (RR), O_2–levels (in %), O_2-saturation levels in the blood (SpO_2, in %), pulse rate (PR), and sevoflurane concentrations (vol%).

Fig. 5.4 A chicken fixed in the raised stereotaxic apparatus with the first cement layer holding the probe and the head block tether.

Fig. 5.5 Microdialysis system and chicken. (a) Infusion pump (1) with swivel arm (2), swivel (3), spring tether guiding the tubing (4), and dialysate vial (5).(b) Chicken during microdialysis

Fig. 5.6 Steps of connecting the probe to the microdialysis infusion system: (a) Metal casing unscrewed with the spiral spring connected to the head block tether. (b) PE tubing guided through the cylinder and connected to the inlet (left, green) and outlet (right, yellow) with Gilson connection pieces. (c) Metal casing screwed on the base. (d) Tape covered the PE tubing. (e) Overview photo of the restrained chicken during connecting of the probe to the microdialysis system with help of (f) the customized blue restraint jacket with the small drawing showing the front view of the jacket with 2 Velcro strips on the back and the larger drawing depicting the sizes (in mm), the compartments for the shoulders (gray stripped sections), and the straps with Velcro (length 300 mm) on the end.

Fig. 5.7 Representatives of (a) chicken and (b) rat dialysate chromatograms. Monoamines and metabolites are measured on separate columns and detector cells. Peak areas represent concentrations (in nM). After calculations using standard curves, 5-HT concentration in chicken dialysate in this example was 3.6 nM and 45.8 nM for 5-HIAA; 5-HT concentration was 0.22 nM and 5-HIAA was 54.9 nM in rat dialysate. Left panels show monoamines: serotonin (5-HT) and dopamine (DA). Right panels show metabolites: 5-hydroxyindoleacetic acid (5-HIAA); 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA).

Stereotaxic surgery

Fixing the chicken in stereotaxic instrument

In the raised stereotaxic instrument, the neck and trachea were at an angle with low risk for respiration to be interrupted. The bird's head was fixed within ear bars (blunted type). Importantly, the ear canal had a turn at a certain depth, thus while the head appeared to be fixed, it was possible that the ear bars had to be a few millimeters deeper for correctly fixing the head. Each distance (read of the ear bars) was noted down to compare between chickens. Also important was that the beak was perpendicular to the ear bars. Then, the Chicken/Duck adaptor was installed in the stereotaxic instrument by sliding the mouth bar in the beak between the upper and lower mandibles while keeping the corners of the mouth bar visible and centered. With this step, it was checked whether the breathing tube remained free from obstructions and whether breathing and other vital parameters were normal. Finally, the nose bar was placed in position on the upper mandible. Note that adult chickens of an age of 23 weeks had a large comb on the head. The nose bar was exactly at the level of the base of the comb on the upper mandible. Still, the nose bar was fixed very tightly, as this guaranteed the angle of the head. As mentioned before, the angle of the adaptor was checked with the custom-made ruler to see whether the tip of eye was in a horizontal line of the tip of the mouth bar (see Fig. 1. pg XII in (Puelles et al., 2007).

Preparation of the head

The feathers on top of the head were plucked by rapidly pulling small plucks of feathers opposite to the direction of growth starting from the back of the head moving to the comb. This was done after verifying that the birds' vital parameters were normal and the bird was well-sedated. The scalp was disinfected with 70% alcohol. Then, the skin was vertically incised in a straight line (about 3 cm) with a small sharp scissor. Local anesthesia was used on the skull membranes (xylocaine (2%) and adrenaline). The analgesic fluid was removed with cotton pads after a few minutes. The membranes were further pushed aside with help of cotton swabs and four bulldog clamps (Lawton, Tuttlingen, Germany) were placed on the edges of the skin. A raster was carved in the clean and dry skull with the tip of a scalpel for better adhesion of the cement, later on. With this latter step and the cutting of the skin, small bleedings could occur. A small piece of Willospon® gelatine sponge (Will-Pharma, Zwanenburg, the Netherlands) was used to stanch the bleeding.

Probe installation

Two drill bits (0.6 mm, HM1006, and 1.0 mm, HM and 1010, Meissinger, Neuss, Germany) and a drill (Dremel 300, Breda, the Netherlands) were used to drill the hole that fitted the probe. The location of the probe was marked on the skull before drilling. In our experience, the marked location had to be checked thoroughly from different

angles as the view could be distorted by stereopsis. By first drilling a little dip at the mark, it was checked whether the planned hole really fitted the AP and ML coordinates of the probe. One should be careful not to damage the duramater when drilling the hole. Remarkably, large individual differences were found for the width (or thickness) of the skull, thus this step had to be executed with caution. A surgical microscope was necessary to check whether the probe could pass the hole without touching any skull edges. It was not uncommon for the hole to fill up with cerebrospinal fluid. This was removed with the tip of a piece of tissue. The tip of the probe was then placed inside the hole just touching the dura to determine the DV coordinate. Importantly, our probes had a fused silica shaft that could easily break the dialysis membrane when put under pressure. Before proceeding with implantation of the probe, four stainless steel skull screws (1x3 mm RVS; Albema Robema, Helmond, the Netherlands) were screwed in the outer layer of the skull in order for the cement to better adhere. Two screws were positioned anterior and two posterior to the probe hole. It was found that the screws, due to the porosity of a birds' skull, fixed better if the screws' diameter exceeded the diameter of the holes (screws with a diameter of 1.0 mm (for anterior) and 1.2 mm (for posterior) in 0.6 mm holes).
The dura was pierced with a needle (25 G * 5/8 inch, Terumo Europe N.V., Leuven, Belgium) prior to insertion to prevent damaging of the dialysis membrane (Benveniste and Hüttemeier, 1990). Then the probe was inserted in the brain tissue.

Probe fixation and protection

The microdialysis probe is fixed with methyl methacrylate dental cement (Vertex-Dental, cold-curing acrylic denture repair material, Zeist, the Netherlands) for several layers. A first layer of cement was put over the screws and stabilized the probe (**Fig. 5.4**). Also, a head block tether (Instech Laboratories, Inc., Plymouth, Pennsylvania, USA) was imbedded in this layer acting as a connection bases for the tubing during actual perfusion of the brain area (see section 0 Microdialysis). A second layer of cement attached a metal cylinder base (outer diameter, OD, of 9 mm; height 6 mm). This metal base holds a metal casing (ID 8 mm, OD 10 mm; height 20 mm) that covered the probe (**Fig. 5.6c**). This latter was an important adjustment to the method used in rats as the sharp toenails, head-scratching, and vigorous head-shakes of chickens could be a real hazard to the functionality of the probe without protective measures taken. The metal base (and casing) fitted over the in- and outlet of the probe and the inside of the base around the probe was carefully filled with cement for extra stability of the probe. About half of the screw-thread was imbedded in the cement. In total, about three layers fixed the probe with cement only covering the prepared skull area, not the skin. The casing was screwed on to cover the probe. The probe remained un-perfused until the following morning.

Recovery

While the cement hardened, the last recording of vital parameters, including the body temperature, took place. Subsequently, the ear bars were loosened and removed and the delivery of sevoflurane ceased. Oxygen flow remained at 0.65 l/min for an additional two minutes to speed up recovery. When the chicken started to swallow and react to the tube, the tube and the Doppler device were removed. The bird was then supplied with 100% oxygen delivered via the mask until it was fully awake. As soon as the chicken attempted to sit/stand and opened its eyes, it was put in a clean cage to recover. Microdialysis followed the next day.

MICRODIALYSIS

Microdialysis system with infusion pump

The microdialysis study was performed in conscious freely moving chickens, one day after implantation of the microdialysis probe. The microdialysis system consists out of an infusion pump, a swivel with arm and tubing connecting the pump with the swivel, the probe and vials to collect the dialysate (**Fig. 5.5a**). The infusion pump (KDS220 series, Kd scientific Inc., Holliston, Massachusetts, USA) infused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant rate of 0.02 ml/h. During microdialysis, the flow rate was set to 0.09 ml/h. Each afternoon before testing, the Ringer fluid was renewed. To prevent salt deposits, the system was run with Milli Q if the time between uses was more than 12h. With the option for ten Kloehn syringes, a maximum of 10 animals can be tested at once. Here, three chickens were tested at the same time. PE tubing leaving the infusion pump is connected to a swivel (372/D/22QM, Instech Laboratories, Inc., Plymouth, Pennsylvania, USA) allowing the animal to optimally perform freely moving behavior. A spiral spring tether (Instech Laboratories, Inc., Plymouth, Pennsylvania, USA) connected to the swivel guided the ascending and descending PE tubing to and from the head of the bird. The tubing ran externally on the upper-spring and internally in the lower-spring. The end of the tubing ran through the lid on the vial (TPX short thread vial 32 x 11.6mm, with integrated 0.2ml glass micro-insert, 15 mm top, Grace Davison Discovery Sciences, USA). Before sampling, a test run determined a delay time of 26 min, i.e. the time from probe to vial. The same infusion system was used in adult chickens as in rats (Prins et al., 2011a; van Heesch et al., 2013; van der Stelt et al., 2005), with some adjustments. These adjustments were because a chicken's head traveled a greater distance and with greater force compared to that of a rat, thus the swivel and all connections were at risk of becoming damaged. Therefore, 1) tape was used to secure the PE tubing to the spiral, near the connections, and on the head, see also next paragraph, 2) the rotation angle arm of the swivel was set to the most expansive angle, 3) two spiral springs were taped together to reach the required length

of 65 from swivel to head, 4) the edges of the slot in metal grid was covered with a rubber hose to protect spiral and PE tubing, and 5) the vial was secured to the spiral with elastic bands to prevent it come off after the wagging of the swivel arm during head movements of the chicken (e.g. pecking at food on the floor).

Connecting the probe to the infusion system

The probe on the chickens' head was connected to the infusion pump in the morning, 3 hours before actual sampling. This period was considered necessary for stabilization of the internal environment around the probe tip (Rossomando, 1998). The test flow (with Ringer fluid) was set at a 0.09 ml/h perfusion rate while connecting the microdialysis system to the probe. The steps on how to connect the probe to the microdialysis system are depicted in **Fig. 5.6**. In short, the chicken was restrained with help of a custom-made jacket, modified from a restraint jacket for parrots (AviStraint, Diamond Avian Distributors, Hurdle Mills, North Carolina, USA) (**Fig. 5.6f**). The metal protection casing was unscrewed and the spiral spring tether was attached to the head block tether (**Fig. 5.6a**). Then, the tubing was run through the casing and the ascending and descending tubing was connected to first the inlet than the outlet, respectively (**Fig. 5.6b**). The casing was screwed back on the base (**Fig. 5.6c**) and the tubing was taped for extra protection, because the tubing protruded the casing (**Fig. 5.6d**). The time to connect the animal to the system lasted approx. 8 minutes and was executed by one person.

Collecting dialysate samples

After the stabilization period of 3 h samples were collected every 30 min. Vials contained 15 µl 0.1M HAC and 45 µl dialysate was collected. The vials were collected and stored at -70^{o}C. At a later time point, the analyses of the dialysate samples were injected into a High Performance Liquid Chromatography with Electrochemical Detection (HPLC-EC) instrument for neurochemical detection and analysis. After collecting the final samples, animals were anesthetized with Sevoflurane via the face mask and euthanized by decapitation. The brains were removed and put in formaldehyde for later dissection and probe determination.

Chromatographic conditions

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and dopamine (DA) with corresponding metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Zoeterwoude, the Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 x 50 mm, 3 µm particle size) in combination with detector cell 1, detected monoamines. Column 2 (NeuroSep 115 C18

1 x 150 mm, 3 µm particle size) in combination with detector cell 2, detected the metabolites. The mobile phase for column 1 for chickens consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 9% MeOH, 5 % ACN, 18.5 % methanol and 400 mg/l OSA. In comparison, the mobile phase for column 1 for rats consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 18.5 % Methanol and 400 mg/l OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5 % methanol and 700 mg/l OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. Monoamines were detected electrochemically using µVT-03 flow cells (Antec, Zoeterwoude, the Netherlands) with glassy carbon working electrodes. Potential settings were for 5-HT and DA +0.30 V versus Ag/AgCl and for the metabolites +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec, Zoeterwoude, the Netherlands). The limit of detection was 0.05 nM (S/N ratio 3:1). Absolute extracellular monoamine concentrations are expressed as nM.

RESULTS

Animals and methodological success rate of the surgery

All animals (24 in total) were successfully operated. All surgeries lasted around 81 ± 2 min (mean ± SEM), with one exception. In this particular case, the fixing of the head in the adaptor which required attention in all birds was particularly hard. Due to this difficulty this surgery lasted 120 minutes. When recovering from the (otherwise uncomplicated) surgery, this chicken showed signs indicative of hyperthermia (i.e., heavily panting with open beak and wings held away from the body). The bird was placed in a cool environment and supplemented with extra oxygen and SC fluids to aid recovery from this situation. With these measures, this chicken recovered within 30 min. No further complications occurred throughout the surgical procedure except for some small bleedings that occasionally occurred upon incising the skin. Especially the area between the skull and muscles posterior on the head were prone to bleeding and needed to be handled with care. As mentioned before, small pieces of Willospon® gelatine sponge were used to successfully stop the bleeding.

Anesthesia and monitoring levels

The depth of the anesthesia was easily assessed by the change in parameters such as the RR, CO_2 and O_2 levels. Increased PR, RR and CO_2-levels and at the same time a drop in O_2-levels were indicative for (too) light sedated animals. Occasionally, birds responded with a sudden increase of the PR (about 15%) to either the fixing of the

head or the plucking of the feathers on the head. Upon noting such a response, the administered sevoflurane concentrations were slightly increased (up to 4 vol%) and further actions were upheld until the PR and RR decreased again as an indication of increased anesthetic depth. Well into the surgery, a sudden rise in PR was recorded following the sound of the turned-on drill independent to any actual drilling in the skull. Other monitoring parameters did not deviate significantly at this occasion. In general, within 10 to 15 min after induction parameters became more regular (lower PR, RR and CO_2-levels and higher O_2-levels) with increased depth of anesthesia. At the start of the surgery, the RR was 11 ± 0.5/min and PR was 219 ± 4 beats/min, whereas at the end of the surgery the RR was 7 ± 0.3/min and PR was 188 ± 7 beats/min. CO_2-levels stayed within 20 – 60 mmHg, O_2-levels were on average 99%, and the spO_2-levels were always higher than 98%. Over the course of the surgery, the body temperature declined approximately 1°C, from 41.1 ± 0.1°C to 40.2 ± 0.1°C. Induction (within 10 min) and recovery (within 6 min) from the sevoflurane anesthesia was quick and uneventful. Average maintenance level for the sevoflurane concentrations ranged from 3.5 to 4.0vol%. Animals did not needed manual support of ventilation. Directly after waking up from anesthesia and being placed back into the cage, birds started scratching and shaking their heads. In general, chickens calmed down within 10 min, and began eating and drinking within the first hour after surgery.

Animals and methodological success rate of the microdialysis

Twenty-one of the 24 animals successfully completed the experiment. Unfortunately, three animals developed complications prior to or during the experiment. Of these birds, two had scratched off the cement cap including the probe in the morning of microdialysis. A third bird showed obvious signs of discomfort and malaise (i.e., not eating or drinking and continuously laying down with closed eyes) prior to being connected to the infusion system. For these reasons, these animals were excluded from the experiment.

Just after being attached to the infusion system, most birds appeared restless, vocalized, and were pacing for approximately 15 to 30 minutes. After this time, chickens calmed down and displayed normal behavioral patterns such as eating, drinking, foraging, dust bathing, preening.

Microdialysate

As clearly depicted by the chromatograms, monoamines and metabolites were measurable in chickens dialysate samples (**Fig. 5.7a**). In this figure a comparison is made between the chromatograms of dialysis samples from chickens and rats (**Fig. 5.7a** and **Fig. 5.7b**). Where a clear separated 5-HT peak is visible in both animal species, the DA peak of chicken dialysate (expected retention time 4.03 min) is obscured by others peaks complicating the analysis of DA concentrations in these

animals. On the other hand, the DA metabolites DOPAC and HVA could be detected, as well as the 5-HT metabolite 5-HIAA.

DISCUSSION

The current paper describes a novel, standardized protocol including adjustments made to make the rats' protocol and system suitable for large adult chickens. The most obvious adjustments made to the standard protocol for rats (Prins et al., 2011a; van Heesch et al., 2013; van der Stelt et al., 2005) were 1) the raised stereotaxic apparatus with a chicken adaptor; and 2) the protection of the probe with a metal casing and securing tape. First, the raised stereotaxic instrument ensured that the head of the chicken was easy to reach while respiration was not obstructed. At the same time, the fixing of the head and establishing the correct angle of the head was the most challenging step within the surgeries. As said, the chickens head had to be fixed at an angle of 43^{o} to bring the skull in the horizontal plane (Puelles et al., 2007). The chicken adaptor enabled a very precise adjustment, although, as describe, the fixing was complicated by the large comb of the adult chicken which covered part of the upper mandible exactly at the level where the nose bar of the adaptor rested on. Despite the tightly fixed nose bar no obvious bruising of the comb were observed afterwards. Second, the protection of the probe with the metal casing worked very well. None of the probes suffered any damage and all animals were connected to the infusion system, except for two chickens that scratched off the cement cap holding the probe in the night before the microdialysis. Probably their nails hooked behind an edge of the cement. This shows the importance of generating a smooth transition between the layers cement and the skull and that the skull is dry before applying the cement. Besides the protection of the probe, the PE tubing and the vials were also extra secured with tape and elastic bands to prevent shifting or detachment of the connections. In addition, the length of the PE tubing and associated length of the spiral guiding the PE tubing depends on height of cage. Importantly, there is a trade-off between the required height of the cage and the length of the tubing. Namely, the longer the length of the tubing, the more monoamines are broken down before reaching the vial. Taking this in consideration, the length of the spiral was made 65 cm with a height of 40 cm for the cage.

The HPLC system detected 5-HT and metabolites in chickens' dialysate. The peak of dopamine could not be clearly discriminated from other peaks in the chickens' chromatogram, since dopamine is surrounded or overlapped by peaks of unknown substances. More method development is required before dopamine can be measured reliantly. However, serotonin (5-HT), its metabolite 5-HIAA and the DA-metabolites DOPAC and HVA concentrations in chickens were well-measurable.

Since this was the first time that microdialysis was performed in adult chickens, the exact duration of the surgeries was difficult to estimate beforehand. Other than the surgeries in young chickens (Zachar et al., 2012; Alam et al., 2012a) but

comparable to our method in rats (Prins et al., 2011a; van Heesch et al., 2013; van der Stelt et al., 2005), we chose to use gas inhalant to control the duration and depth of the anesthesia during surgery. Sevoflurane inhalant provided a fast sedation and fast recovery. Together with monitoring a variety of life-support parameters, this attributed to the 100% survival rate of the surgeries for probe installation in adult chickens. During surgery, large skull width variances were seen. Thus the drilling of the probe hole was executed with care. Also, animals seemed to respond to the drilling by displaying an increased PR, although the PR rose independently of any drilling actions. Probably the animals responded to the sharp loud noise produced when the drill was turned on. Large differences were also seen in the response of the chickens after the probe was attached to the infusion system in the morning of microdialysis. Some animals displayed clear flight behavior (pacing, flapping and attempting to escape) when noticing the metal spiral now hanging in the cage (as it was connected to the head). Other animals seemed less disturbed by this. This shows that the installed stabilization period of 3h is not only necessary for stabilization of the internal environment around the probe, but this time is also necessary for the chickens to become habituated to the new situation.

Performing microdialysis surgery in adult chickens is limited by another important factor: there is no brain atlas currently available with coordinates of the adult brain of chickens. It was not our intention to suggest here that we aimed to create a brain atlas for adult chickens. On the contrary, we used the atlas by Puelles et al. (2007) as a guidance to pinpoint the brain region of interest. However, it was good to realize that the brain of 23 week-old chickens had grown in all directions compared to a young brain. For instance, the corticoseptomesencephalic tract (csm; 4.72 mm anterior to the interaural line) – a good landmark in the brain–was visible in two slides of 400 μm (adult chicken brain) instead of only one slice with a thickness of about 500 μm as depicted in the atlas (young chick brain). This study of brain slices together with pilot microdialysis surgeries followed by making brain slices to track the probe-tract was part of an extensive pilot study performed to determine the exact coordinates of the brain region of interest. We recommend a similar approach if pursuing brain microdialysis in adult chickens.

Microdialysis has the advantages of measuring the direct release of brain monoamines in an individual animal while simultaneously observing the behavior of the tested animals (Westerink, 1995; Gruss and Braun, 1996a; Hasegawa et al., 1994). Some detrimental behaviors, like feather pecking within laying hens, mostly occur at an adult age in chickens (Gilani et al., 2013). Feather pecking behavior is a serious welfare problem in the laying hens industry (Rodenburg et al., 2013). Moreover, feather pecking has been related with serotonergic and dopaminergic differences between high and low peckers (van Hierden et al., 2004; Kops et al., 2013b; Kops et al., 2013a). Considering this, microdialysis provides a new method to gain insight in the neurochemical background of these and other behaviors in adult chickens. With minor adjustments the rat microdialysis system can be made suitable for chickens. The

absence of an atlas with brain coordinates of adult chickens remains an existing challenge, but despite this, microdialysis in adult chickens is very feasible using the protocol we describe in this paper.

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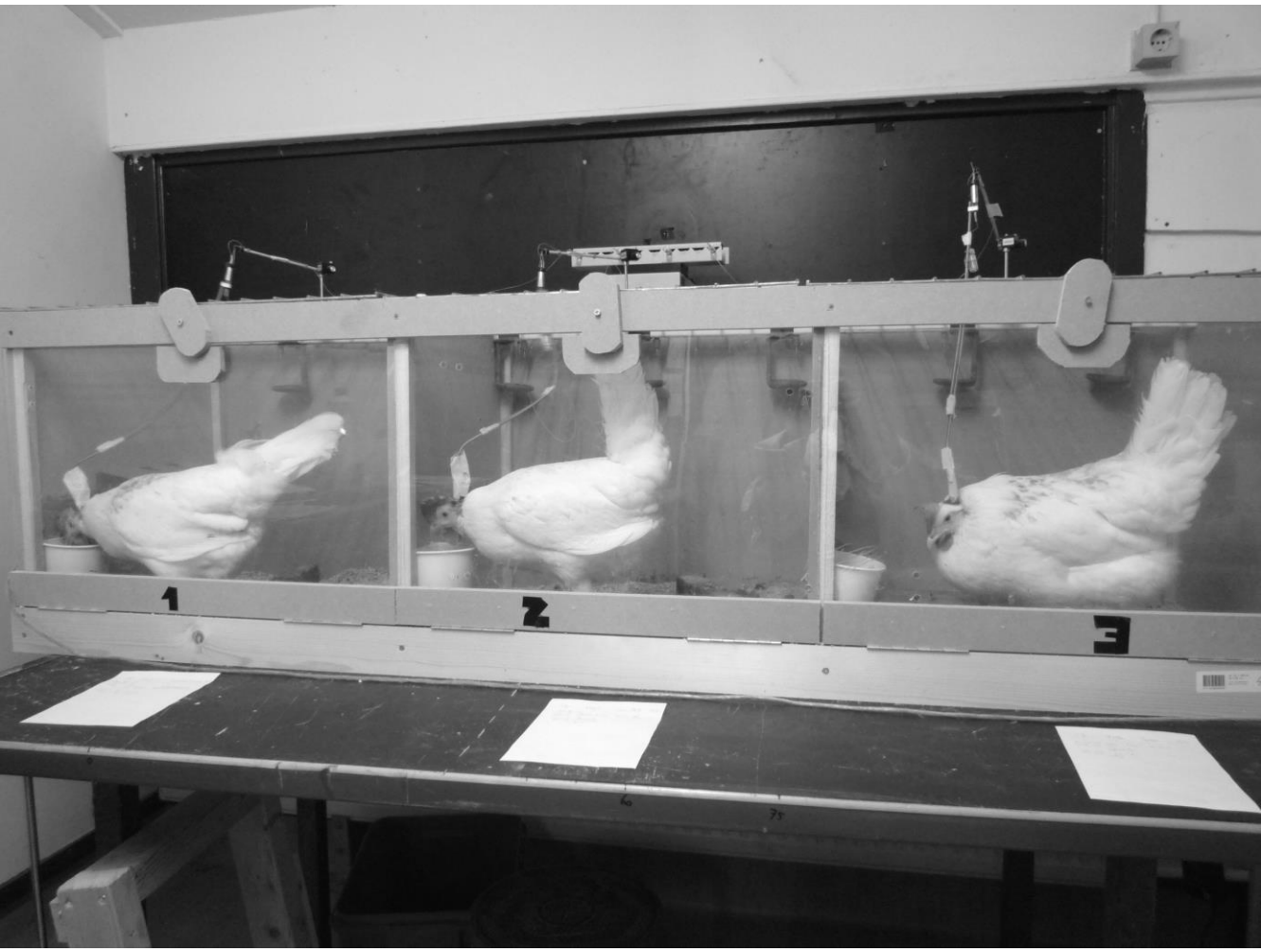
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Chapter 6

Serotonin release in the caudal nidopallium of adult laying hens genetically selected for high and low feather pecking behavior: an *in vivo* microdialysis study

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SUBMITTED

ABSTRACT

Severe feather pecking (FP) is a detrimental behavior causing welfare problems in laying hens. Divergent genetic selection for FP in White Leghorns resulted in strong differences in FP incidences between lines. More recently, it was shown that the high FP (HFP) birds have increased locomotor activity as compared to hens of the low FP (LFP) line, but whether these lines differ in central serotonin (5-hydroxytryptamine, 5-HT) release is unknown. We compared baseline release levels of central 5-HT, and the metabolite 5-HIAA in the limbic and prefrontal subcomponents of the caudal nidopallium by *in vivo* microdialysis in adult HFP and LFP laying hens from the ninth generation of selection. A single subcutaneous d-fenfluramine injection (0.5 mg/kg) was given to release neuronal serotonin in order to investigate presynaptic storage capacity. The present study shows that HFP hens had higher baseline levels of 5-HT in the caudal nidopallium as compared to LFP laying hens. Remarkably, no differences in plasma tryptophan levels (precursor of 5-HT) between the lines were observed. D-fenfluramine increased 5-HT levels in both lines similarly indirectly suggesting that presynaptic storage capacity was the same. The present study shows that HFP hens release more 5-HT under baseline conditions in the caudal nidopallium as compared to the LFP birds. This suggests that HFP hens are characterized by a higher tonic 5-HT release.

INTRODUCTION

Severe feather pecking (FP) is the pecking at and pulling out of feathers of conspecifics. This detrimental behavior causes welfare problems in laying hens and has multifactorial causes (Savory, 1995; Rodenburg et al., 2013; Gilani et al., 2013). Genetic studies have shown a moderate heritability of FP (Rodenburg and Koene, 2003; Kjaer and Sørensen, 1997) with genetic variations in several genes of the monoaminergic systems that seem to be related to FP behavior (Biscarini et al., 2010; Flisikowski et al., 2009; Brunberg et al., 2011). From neurobiological and pharmacological studies there is indeed a growing body of evidence on the involvement of brain monoamines such as serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) in the propensity to develop FP (Bordnick et al., 1994; van Hierden et al., 2004; van Hierden et al., 2005; Biscarini et al., 2010; Van Hierden et al., 2002; Kops et al., 2013b; Kops et al., 2013a). Comparing brain monoamine levels in young chickens from commercial lines selected on production traits (e.g. egg size and egg quality), unintentionally also differed in levels of FP, revealed that the young chickens of the line with higher FP levels had lower 5-HT and DA turnover ratios than the line with lower FP (Van Hierden et al., 2002). Treatment of these young chickens with a tryptophan-rich diet (van Hierden et al., 2004) or pharmaceutical D2 receptor antagonist such as haloperidol (Kjaer et al., 2004) was very effective at reducing gentle FP ratios by increasing brain 5-HT and DA levels. In contrast, more gentle FP incidences were recorded after decreasing 5-HT levels by inhibiting 5-HT release via a 5-HT1A autoreceptor agonist (van Hierden et al., 2004). Thus gentle FP may be related to low turnover of central 5-HT and DA as shown in the rostral forebrain of young chickens. It should be noted, though, that severe FP is mostly prevalent at an adult age (Gilani et al., 2013; Newberry et al., 2007). Differences in brain monoamine levels were inconsistent in adult chickens of commercial lines selected for traits other than FP, that coincidentally also affected FP (Uitdehaag et al., 2011; Cheng and Fahey, 2009; Craig and Swanson, 1994). In 1997, Kjaer et al. (Kjaer and Sørensen, 1997) started genetically selecting chickens on their individual display of severe FP behavior. This resulted in experimental lines called the high (HFP) and low (LFP) feather pecking selection lines. Next to strong divergences in FP ratios in the third and following generations (Su et al., 2005; Kjaer et al., 2001), the HFP compared to LFP were more motivated to eat feathers (Harlander-Matauschek and Feise, 2009) – a behavior related to FP (Rodenburg et al., 2013; Bennewitz et al., in prep.) – and differed in gut flora (Meyer et al., 2013) and had also increased locomotor activity in their home cage as compared to LFP (Kjaer, 2009).

The objective of the current study was to measure the release of monoamines in the extracellular synaptic cleft in the caudal nidopallium of adult HFP and LFP hens by *in vivo* microdialysis. The nidopallium is a large associative area in the chickens' forebrain with a potential role in the guiding of motor actions and decision making (Jarvis et al., 2005; Reiner et al., 2004; Güntürkün, 2005). The caudolateral nidopallium (NCL) receives, more than the caudocentral nidopallium (NCC), input

from monoaminergic systems and serves frontal-like executive functions (Güntürkün, 2012). The NCC displays a limbic connectivity (Shanahan et al., 2013; Atoji and Wild, 2009). Both NCL and NCC have reciprocal projections to the arcopallium intermedium, a somatosensory area, and the arcopallium mediale, a limbic region (Metzger et al., 1998; Kröner and Güntürkün, 1999; Atoji and Wild, 2009). Both nidopallic regions contain serotonergic (Metzger et al., 2002) and dopaminergic afferents (Durstewitz et al., 1998) and receptors (Durstewitz et al., 1999b; Herold et al., 2012b; Herold et al., 2011b). In contrast to taking samples of brain tissue (Cheng and Fahey, 2009) or measuring 5-HT blood concentrations (e.g. (Cheng et al., 2001a; Buitenhuis et al., 2006) *in vivo* microdialysis allows the measurement of the extracellular monoamine release within a short timeframe in a particular brain area of conscious freely moving animals. Appropriate central monoamine release is essential for stimulation of pre- and postsynaptic monoamine receptors corresponding with adequate stimulation of the second messenger systems or target organs (see review on 5-HT metabolism by (Strüder and Weicker, 2001). Although microdialysis is used to study, for instance, the role of monoamines in imprinting (Metzger et al., 1998; Tsukada et al., 1999; Gruss et al., 2003) and feed intake (Alam et al., 2012a) in young chickens, as far as we know, microdialysis has never been performed in adult chickens. A second objective was to compare blood plasma concentrations of tryptophan (precursor of brain 5-HT (Bongiovanni et al., 2010)) between the HFP and LFP lines to establish whether potential line differences in the release of monoamines in the brain might have a peripheral cause (e.g. by diet) or whether there is evidence for an altered synthesis and/or release in the brain. Here we investigate whether divergent selection for FP produces differences in serotonergic neurotransmission in the forebrain of adult laying hens.

MATERIAL AND METHODS

Ethical statement

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University, the Netherlands, and found to be in accordance with Dutch legislation on the treatment of experimental animals, the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

Animals and housing

White leghorn hens from the 9th generation of divergently selected lines, the HFP and LFP, were used. Details regarding the selection procedure have been described previously (Su et al., 2005; Kjaer et al., 2001). Eggs of both HFP and LFP birds were brooded and after hatch, the one-day old female chicks received a health check followed by a neck tag with a color/number combination for identification. In total 84

female chicks were distributed over 12 pens (42 chicks/line; *n* =7/pen). Birds were not beak-trimmed. The chicks were housed in pens with a concrete floor (1.9 by 1.2 m) covered with paper (first 7 weeks) or sawdust (after week 7). Water and a commercial mash diet were provided *ad libitum*: a starter diet (week 1-5), a grower diet (week 6-16) and a layer diet (from week 17 onwards). Each pen had a 50 cm high perch installed and a lower perch (a block of wood) in the first seven weeks. In week 8, each group was reduced by one chicken (used for another experiment). By that time, three chicks turned out to be male and 4 chicks had died within the first week. Therefore, the total group size was 65 animals (LFP: *n* = 32; HFP: *n* = 33). Continuous light was given the first week, and then 18 h of light (week 2) followed by 13 h (week 2 - 3), and 10 h of light (week 4 – 15). From 17 weeks of age onwards, the light period was extended by 1 h per week, until the birds had 16 h of light (2.00 am – 6.00 pm) at 23 weeks of age.

HPLC-ECD determination of large neutral amino acids (LNAA) in blood plasma

At 17 weeks, blood taken from the wing vein was collected in a 4 ml EDTA tube and put on ice. Samples were centrifuged and 200µl plasma was put in a 1 ml serum tube and stored at -70^{o}C until analysis. Large neutral amino acids (LNAA), such as tryptophan (TRP), l-valine (Val), I-methionine (Met), leucine (Leu), I-isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), and the internal standard I-norleucine (NLeu) were detected simultaneously using a ultra-high performance liquid chromatography (UHPLC) with electrochemical detection using an Alexys 110 LC-EC analyzer (Antec, Zoeterwoude, the Netherlands). The system consisted of two pumps, one autosampler with a 1.5 µl loop, a column (Acquity UPLC HSS T3 1.0 x 50 mm, 1.8 µm particle size, Waters, Milford USA), a µVT-03 detector flow cell with glassy carbon working electrode (potential setting +0,85 V versus Ag/AgCl). The column and detector cell were kept at 40°C in a column oven. Stock solutions of the amino acid were prepared in Milli-Q water and stored at -70 °C. To 20 µl of plasma 80 µl 100% methanol was added and subsequently vortex mixed. Then 20 µl 0.5 mM NLeu was added and vortex mixed. After 10 min on ice the samples were centrifuged during 10 min at 15000g. Subsequently 20 µl of the supernatant was added to 60 µl of 0.05M sodium borate buffer pH 10.4, mixed and pipetted into autosampler vials. During analysis the samples were kept at 4 °C in the autosampler. Primary amino acids in the sample were derivatized pre-column (Smith and Sharp, 1994) using a reagent consisting of 37.5 mM o-Phtalaldehyde (OPA) (Pickering Laboratories, USA), 50 mM sodium sulphite, 90 mM sodium borate buffer pH 10.4. This reagent was prepared by mixing a 0.75M OPA solution (prepared in methanol) with a 1M sodium sulphite solution (in Milli-Q water) and a 0.1 M sodium borate buffer pH 10.4 (mixing ratio 1:1:18). The derivatisation was performed automatically in-line using the autosampler. A 9 µl sample was mixed with 0.5 µl reagent just prior to the analysis. Separation was

achieved using mobile phase A (50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, pH 4.5, 8% acetonitril, 10% Methanol). As soon as the compounds of interest were completely detected a step gradient using mobile phase B (50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, pH 4.5, 60% acetonitril) was applied to rinse the column removing any late eluting compounds. The flow rate was set at 200 µl/min. The chromatogram was recorded and analyzed using a Clarity data system (Antec, Zoeterwoude, the Netherlands). Concentrations of LNAAs in the sample chromatograms were calculated using a calibration curve and corrected for recovery variations using the internal standard. The limit of detection for TRP was 50 nM (signal to noise ratio 1:3). Met and Val peaks overlapped. Concentrations were depicted in µM (is mol/l). The TRP/LNAA ratio was determined by dividing TRP concentrations by the sum of the other LNAA (Tyr + (Met + Val) + Ile + Leu and Phe) (Fernstrom, 2012).

Chemicals and drugs

TRP, Tyr, Val, Met, Leu, Ile, Phe, NLeu were obtained from Sigma Aldrich, USA. Citric acid, phosphoric acid, ethylenediamine-tetraacetic acid disodium salt (EDTA), sodium hydroxide, potassium chloride, and, 1-Octanesulfonic acid sodium salt (OSA) were obtained from Acros Organics, Belgium. Boric acid was obtained from Merck, Germany. Acetonitril and Methanol were obtained from Biosolve BV, the Netherlands, and o-Phtalaldehyde (OPA) from Pickering Laboratories, USA. Sevoflurane (SevoFlo, Abbott Animal Health, Chicago, Illinois, USA) was used to induce a rapid and effective general anesthesia. Carprofen (Rimadyl, 50 mg/mL, Pfizer Animal Health, Capelle a/d IJssel, the Netherlands; 5 mg/ kg BW SC) and butorphanol (Dolorex 10 mg/mL, Merck Animal Health, Schiphol-Rijk, the Netherlands; 1 mg/kg BW SC) were used as general analgesia. Local analgesia on the skull was alfacaine (2%) with adrenaline (Alfasan Nederland BV, Woerden, the Netherlands). D-fenfluramine (Sigma Aldrich, USA) was dissolved in saline and administered after baseline measurements during microdialysis, into the inguinal region at a dose of 3.0 mg/kg BW SC (diluted to a volume of 0.5 ml/kg). D-fenfluramine is a serotonin releaser and serotonin reuptake blocker and induces the release of serotonin from the presynaptic neuron and blocks the re-uptake of serotonin (Kleven and Seiden, 1989; Wang et al., 1999; Rothman et al., 2003).

Microdialysis

Habituation

At 23 weeks of age, two hens per pen were randomly selected for microdialysis (n=12/line). Prior to surgery, each selected hen was habituated for 3 to 5 days in the microdialysis room adjacent to the operating room. There, hens were individually kept in wooden boxes (60 x 30 x 40 cm) with steel mesh as rooftop and a Perspex window

in the front and one in the partitions allowing visual contact with the neighboring hen. Light was controlled by a time switch (2.00 am - 6.00 pm). A radio played during light hours and animals also became habituated to humans walking close by their cages. Temperature in the habituation room was on average (± SEM) 18.7 ± 1.4°C with an average humidity of 60.4 ± 9.2 %. In the morning of surgery, the chickens were put on feed restriction (Curro, 1998) and birds were weighed. Water remained continuously available via the drinking nipples in the cage.

Surgery

Hens were anesthetized with a mixture of sevoflurane (7-8 l/min) and oxygen (0.8 l/min). When sedated, substances of analgesia were administered subcutaneously in the inguinal region. Body weight was on average 1.2 ± 0.3 kg. Body temperature was taken before and after surgery. Oxygen (O_2) flow was kept on 0.65 l/min for the entire procedure with a lower maintaining sevoflurane gas flow (3 - 4 l/min) administered via an intubation tube. Each hen was stereotaxically implanted with a microdialysis probe (MAB 4.11.2CU, 2 mm membrane length, Microbiotech, Stockholm, Sweden) aimed to cover the area of NCC and NCL as higher-order limbic and executive structures (**Fig. 6.1**). The head was fixed in a 6 inch (15.2 cm) raised stereotaxic apparatus (model 902, David Kopf instruments, Tujunga, California, USA) and a chicken/duck adapter (model 917, David Kopf instruments) with the adaptor slide (model 1246, 45 Degree Adaptor Slide, David Kopf instruments, Tujunga, California, USA) set to an angle of angle of 43° to bring the skull in the horizontal plane. The stereotaxic coordinates were determined following the brain atlas of a two-week old chick by Puelles et al. (Puelles et al., 2007). The absence of a brain atlas for adult chickens provided an uncertainty of the probe location, but a pilot study (described shortly in **Chapter 5**) provided the following coordinates: A; anterior to interaural line +6.0 mm, L; lateral to the interaural line +7.3 mm, V; ventral from the dura mater -6.3 mm. Probes were anchored on the skull with anchor screws and dental cement (methyl methacrylate dental, Vertex-Detal, Zeist, the Netherlands). To prevent chickens from damaging the probe by head scratching, the probe was also covered by a metal casing (aluminum) with a base imbedded in the cement. After implantation, hens were housed individually and placed in the microdialysis room until the end of the experiment. Three hens were subjected to surgery per day.

Microdialysis study

The microdialysis study was performed in conscious freely moving hens, one day after implantation of the microdialysis probe. A pump (KdScientific Pump 220 series, USA) perfused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant flow rate of 0.02 ml/h. During microdialysis, the flow rate was set at 0.09 ml/h. At 7.45 am, hens were connected to a channel swivel (type 375/D/22QM) which allowed them to move freely. At 11.00 am, about three hours

after connection, 30-min samples were manually collected in vials containing 15 µl of 0.1 M acetic acid and frozen at -70 °C until analysis with HPLC. From 11.00 am until 1.00 pm four baseline samples were collected. Hereafter, d-fenfluramine was injected and, at 17.00 pm, the final sample (12 in total) was taken. Thereafter animals were culled and the brains were dissected and immediately stored in formaldehyde (4%) for later investigation of probe localization.

Chromatographic conditions

Microdialysis samples were stored at –70 °C until analysis. The neurotransmitter serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), respectively, were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Zoeterwoude, the Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 x 50 mm, 3 µm particle size) in combination with detector cell 1, detected 5-HT. Column 2 (NeuroSep 115 C18 1 x 150 mm, 3 µm particle size) in combination with detector cell 2, detected the metabolite. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 9%MeOH, 5% ACN and 400 mg/l OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5 % methanol and 700 mg/l OSA. Both mobile phases were pumped at 50 µl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 µl was injected simultaneously onto each column. The neurotransmitter 5-HT was detected electrochemically using µVT-03 flow cells (Antec, Zoeterwoude, the Netherlands) with glassy carbon working electrodes. Potential settings were for 5-HT +0.30 V versus Ag/AgCl and for the metabolite 5-HIAA +0.59 V versus Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec, Zoeterwoude, the Netherlands). The limit of detection was 0.05 nM (S/N ratio 3:1). Absolute extracellular monoamine concentrations are expressed as nM. Besides measuring the absolute extracellular concentrations of 5-HT and 5-HIAA, also the response to d-fenfluramine was measured. It was not possible to measure DA concentrations in the chromatogram, because other molecules in the sample disturbed the DA-peak.

Histology

Dissected brains were quickly stored in formaldehyde until verification of the probe localization. Two days before brain slicing, the brains were placed in 30% sucrose solution. Probe placements were verified on 60 µm cresyl violet stained sections obtained with the frozen technique.

STATISTICAL ANALYSES

Microdialysis

Effects of line (LFP vs. HFP) on baseline monoamine and metabolite were analyzed by repeated measures ANOVA with time (4 levels: -90 min, -60 min, -30 min, and 0 min) as within-subject factor and line (LFP or HFP) as between-subject factor. Post-injection data were compared in repeated measures ANOVA with time (8 levels: 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min, and 240 min) as within-subject factor and line (LFP or LFP) as between-subject factor. When the assumption of sphericity was violated, the results were corrected by the Greenhouse-Geisser procedure. All data were analyzed using the SPSS 20 software statistical package. For the analyses, only the data of hens with the probe localized in the area of interest (based on the histological study, see below) were included.

Large neutral amino acids concentrations in blood plasma

Mean levels of the large neutral amino acids (LNAAs) in blood plasma in the LFP and HFP were analyzed with use of independent t-tests. Blood was taken when animals were 17 weeks of age. At that age, the group consisted of 65 animals (LFP: n = 32; HFP: n = 33,). A line comparison was made within this former group. Also, a line comparison was made within the subset of selected animals based on the histological study after microdialysis (LFP: n = 5; HFP: n = 6).

RESULTS

Histology

Data of three hens was excluded before histology took place: two hens (of the LFP line) removed their cement caps before the actual microdialysis started, whereas a third hen (of the HFP line) had to be culled because of surgical complications. Due to this, histology to determine the probe placement was performed in 21 brains (LFP: n=10; HFP: n=11). The probes were aimed at the borderline of the NCC and the NCL (**Fig. 6.1**). Birds with a probe localized outside the region of interest (anterior distance from the zero point: 2.56 mm – 2.08 mm) were excluded from the dataset. This held for 10 animals in total (n=5 per line). Consequently, the group size was reduced to eleven in total (LFP: n = 5; HFP: n = 6).

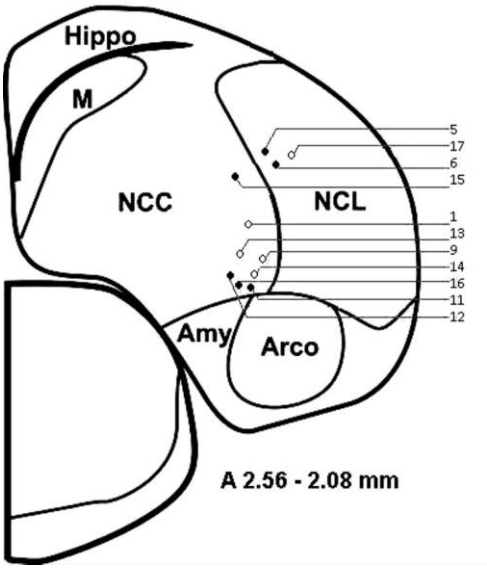

Fig. 6.1 Probe placements in the left caudal nidopallium illustrated in a schematic coronal section of a 23 week-old chicken brain. The representation of the brain areas in adult chickens is based on tracing and immunohisto-chemistry studies in chickens and pigeons (Jarvis et al., 2005; Reiner et al., 2004; Güntürkün, 2005; Metzger et al., 1998; Atoji and Wild, 2009) and on the (young) chicken brain atlas by Puelles et al. (Puelles et al., 2007). The anterior distance from the zero point is labeled (2.56 mm to 2.08 mm following (Puelles et al., 2007). Open circles = LFP; closed circles = HFP. Abbreviations: Amy = amygdala; Arco = arcopallium; Hipp = hippocampus; M = mesopallium; NCC = caudocentral nidopallium; NCL = caudolateral nidopallium

Serotonergic dialysate concentrations

Baseline serotonin and metabolite levels in the caudal nidopallium

Fig. 6.2 shows the extracellular levels of 5-HT, 5-HIAA and 5-HT turnover. Repeated measures ANOVAs on the first four baseline measurements revealed significant line differences for 5-HT ($F_{(1,9)}$ = 17,34; P = 0.02), with HFP having higher 5-HT levels compared to LFP. Lines did not differ in baseline 5-HIAA concentrations ($F_{(1,9)}$ = 0.19; P = 0.68). HFP had a significant lower 5-HT turnover ratio compared to LFP at baseline ($F_{(1,9)}$ = 5.31; P = 0.047). There were no significant time x line interactions and no effect of time on the absolute mean baseline values, with the exception of 5-HIAA levels ($F_{(1,3)}$ = 3.19; P = 0.04) which slightly decreased over time.

Effect of d-fenfluramine on monoamine and metabolite levels in the caudal nidopallium

The d-fenfluramine-response from time point zero onwards for 5-HT, 5-HIAA, and turnover is shown in **Fig. 6.2**. Time since administration of d-fenfluramine affected levels of 5-HT ($F_{(1,1.343)}$= 12.821; P =0.002, ε = 0.192) and 5-HIAA ($F_{(1,2.982)}$ = 4.763; P = 0.01, ε = 0.731), although lines did not differ (5-HT: $F_{(1,9)}$ = 0.002, P =0.96; 5-HIAA: $F_{(1,9)}$ = 2.466, P = 0.151). There was no time x line interaction ($F_{(1,7)}$ = 0.019, P =1.00) or line effects ($F_{(1,9)}$ = 0.002, P = 0.963) for 5-HT. Time affected post-d-fenfluramine 5-HT turnover ratio ($F_{(1,7)}$ = 12.821, P = 0.00).

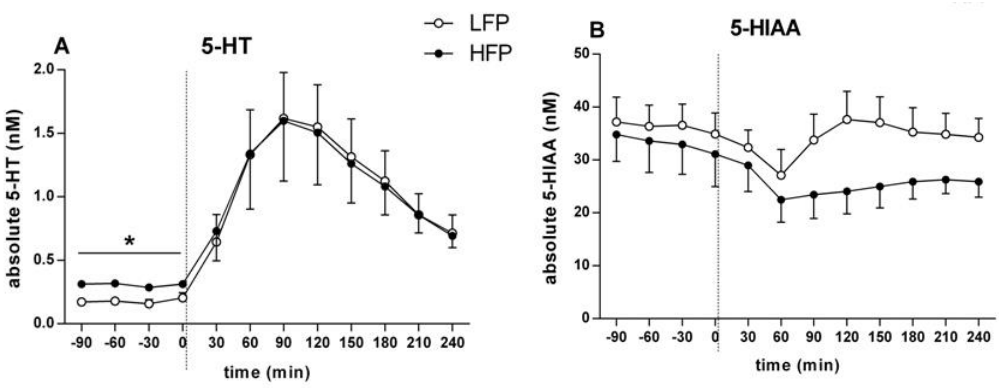

Fig. 6.2 Measuring 5-HT and 5-HIAA by *in vivo* brain microdialysis in the caudal nidopallium of adult LFP and HFP hens. 5-HT (A) and 5-HIAA (B) in the caudal nidopallium were measured under basal conditions (4 time points, -90 min – 0 min) and after d-fenfluramine injection (8 time points, 30 min – 240 min) (left and right from the dotted line). LFP in open circles (n=5) and HFP in closed circles (n=6). Mean (± SEM) values for monoamines, **P*<0.05

Large neutral amino acids concentrations in blood plasma

No line effects were found for the other LNAA levels measured or the TRP/LNAA ratio in this subset of animals, see **Table 6.1**. When comparing between all HFP and LFP hens (65 in total), lines differed in Tyr ($F_{(1,63)}$ = 2.33, P = 0.000), with higher levels in LFP (152.12 ± 5.36) compared to HFP (120.47 ± 3.37) Moreover, line differences were found for Phe ($F_{(1,63)}$ = 0.45, P = 0.011), and lines tended to differ for TRP ($F_{(1,63)}$ = 1.00, P = 0.082). Compared to HFP, LFP had higher levels for Phe (HFP: 76.5 ± 2.03 vs. LFP: 83.6 ± 1.79) and tended to have higher levels for TRP (HFP: 86.31 ± 2.30 vs. LFP: 92.36 ± 2.53).

Table 6.1 LNAA levels (mean ± SEM) in blood plasma in LFP (*n*=5) and HFP (*n*=6) hens

LNAA	LFP	HFP	*P*-value
TRP/LNAA[1]	0.15 ± 0.01	0.15 ± 0.01	0.775
TRP	82.7 ± 3.90	81.7 ± 4.93	0.763
Met + Val	104.4 ± 0.82	102.0 ± 5.28	0.843
Ile	86.2 ± 14.68	83.90 ± 7.92	0.886
Leu	168.9 ± 15.45	161.2 ± 8.96	0.666
Phe	82.2 ± 2.49	77.1 ± 4.27	0.457
TYR	147.3 ± 11.45	122.0 ± 9.17	0.115

[1]Ratio of tryptophan to the sum of the other LNAA [TRP/ (Met+Val+Ile+Leu+Phe+TYR)]

DISCUSSION

Serotonergic differences in lines selected for and against feather pecking

This microdialysis study in adult hens from the experimental selection lines demonstrated that HFP hens had a significant higher baseline 5-HT release in the caudal nidopallium as compared to LFP hens. Strikingly, the baseline 5-HT concentration had very little variance within line. Note that the 'punch' method used in most other studies, i.e. analysis of brain tissue samples as a whole, does not allow for distinction between the 5-HT and 5-HIAA concentrations in the presynaptic neuron and the extracellular matrix. Here, the release of monoamines within one specific brain area was targeted by *in vivo* microdialysis. To our knowledge, this is the first time that *in vivo* microdialysis is performed in adult laying hens, although *in vivo* microdialysis has been performed in young chickens (25 days of age) (Metzger et al., 1998; Tsukada et al., 1999; Gruss et al., 2003);(Alam et al., 2012a).

The present study demonstrates that HFP hens have higher baseline 5-HT concentrations compared to LFP birds. Several neurobiological factors may be responsible for the observed increased baseline release of 5-HT. For instance, higher 5-HT release could be caused by 1) more tryptophan (TRP) in the blood available (TRP is the precursor for brain 5-HT), 2) a higher enzymatic activity of tryptophan hydroxylase (TPH; metabolizes TRP to 5-HT), 3) a decreased activity of the serotonin transporter (SERT; facilitates the reuptake of released 5-HT into the presynaptic cell), 4) a lowered monoamine oxidase of type A (MAO-A; metabolizes 5-HT to 5-HIAA), or, finally, 5) altered 5-HT1A autoreceptor activity (is part of the short negative feedback loop located on the presynaptic neuron). Peripheral TRP is a precursor for brain 5-HT (Eisenhofer et al., 2004; Strüder and Weicker, 2001). TRP has to compete with other large neutral amino acids (LNAA) present in the blood to enter the brain via the blood-brain-barrier. Here it is shown that HFP and LFP birds do not differ in their TRP concentrations or in TRP/LNAA ratio. This is confirmed by plasma measurements in an earlier generation of these selected lines (Buitenhuis et al., 2006) and suggests that the higher baseline release of 5-HT cannot be attributed to differences in TRP availability. The synthesis of 5-HT from TRP can, however, be affected by the activity of the enzyme TPH (Strüder and Weicker, 2001). D-fenfluramine induced a dramatic increase in 5-HT concentration in both lines, without differences between the lines. This latter observation demonstrates that the storage capacity in HFP hens probably does not differ from that of LFP hens.

Two more factors important for 5-HT metabolism might be affected by selection on FP, namely SERT and MAO-A activity. SERT located on the membrane of presynaptic neurons facilitates the 5-HT clearance from the synaptic cleft (Blakely et al., 1991; Hoffman et al., 1991) whereafter 5-HT can be stored again in the vesicles for future release. There are indications for SERT-involvement in FP from commercial

selection lines since chickens selected on low mortality (due to low incidences of FP and cannibalism) differed from a control line in peripheral SERT functioning (Bolhuis et al., 2009). Peripheral SERT functioning has some predictive value for the activity of the central reuptake system (Yubero-Lahoz et al., 2012). Besides possible genetic selection effects, the impact of elevated 5-HT levels during life on the receptor activity should not be overlooked. Sustained elevated 5-HT has been recognized, both in the periphery and centrally, to affect not only the receptor sensitivity but also to have a down-regulatory effect on SERT functioning itself (reviews by (Mercado and Kilic, 2010; Ramamoorthy and Blakely, 1999). However, there is no significant difference in the levels of the 5-HT metabolite 5-HIAA to underpin this hypothesis. Concerning MAO-A, absence of differences in 5-HIAA levels also implies that MAO-A activity is not affected in either of the lines. On the other hand, polymorphisms on the gene coding for the MAO-A have been associated with the susceptibility to receive FP (Biscarini et al., 2010), but here, the effect of selection on FP on both SERT and MAO activity remains elusive. The 5-HT_{1A} autoreceptor is part of a short negative feedback loop located on the presynaptic neuron (Caramaschi et al., 2007). Under normal conditions, 5-HT molecules are released in the presynaptic cleft and part of these molecules will reach the 5-HT_{1A} autoreceptor, thereby inhibiting neuronal firing and consequently suppressing 5-HT synthesis. A lower serotonergic neurotransmission caused by an underlying hypersensitive 5-HT_{1A} autoreceptor system has been suggested to be a trait-characteristic of violent rodents, showing escalated aggression (Cheng and Muir, 2007; Caramaschi et al., 2007; De Boer et al., 2003; de Boer and Koolhaas, 2005; Suzuki et al., 2010; Korte et al., 1996; Sijbesma et al., 1991). The relationship between HFP and higher baseline 5-HT neurotransmission found in the present study does not suggest a similar involvement of the 5-HT_{1A} autoreceptors in SFP as for aggression. This supports the hypothesis that feather pecking is different from aggressive pecking (Savory, 1995; Bessei et al., 2013).

Selection on FP behavior and its neurobiological effects

Recently, it was found that chicks from the HFP line walked a longer distance in their home pen than LFP chicks which lead to the suggestion that HFP chicks suffer from a hyperactivity disorder (Kjaer, 2009). These high levels of activity in the HFP may originate from a more activated motor system. Both the NCC and NCL are connected with the medial arcopallium (AM), the n. posterioris amygdalopalii (PoA), the n. taeniae amygdalae (TnA), and intermediate arcopallium (AI) (Jarvis et al., 2005; Reiner et al., 2004; Atoji and Wild, 2009). Whereas AM, PoA, and TnA are considered limbic and homologous to subcomponents of the amygdala, the AI is a somatomotor area with secondary sensory afferents and projections to rhombencephalic motor areas (Zeier and Karten, 1971). Both NCC and NCL contain similar amount of serotonergic fibers and show a high density of 5-HT_{1A} binding sites (Metzger et al., 2002), but the NCL is more densely covered with dopaminergic fibers than the NCC (see pigeon

studies by Durstewitz et al., 1999b; Metzger et al., 1996). It has been described that both increased and decreased levels of 5-HT may lead to enhanced locomotor activity and enhanced reactivity to a novel stimuli and environmental changes (Gerson and Baldessarini, 1980). These behaviors fit the HFP chicks, although these behaviors are usually generated by lower 5-HT levels (Gerson and Baldessarini, 1980).

CONCLUSION

The present *in vivo* microdialysis experiment clearly shows that adult laying hens of the HFP line are characterized by higher baseline serotonin release in the caudal nidopallium as compared to birds of the LFP line. This suggests that a higher tonic 5-HT neurotransmission activity under baseline conditions is a trait-characteristic of HFP hens. This study illustrates that microdialysis in adult laying hens selected for divergences in feather pecking can provide interesting new perspectives on the role of 5-HT in feather pecking.

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Chapter 7

General discussion

FEATHER PECKING AND MONOAMINES

Severe feather pecking (SFP) is abnormal behavior and causes feather damage, which may result in cannibalism in laying hens. It has been hypothesized that the serotonergic and dopaminergic system are involved in the etiology of SFP. Understanding the neural mechanisms of SFP may provide early predictors and indicators of this injurious behavior. The aim of this thesis was to study the role of monoaminergic systems in SFP. In order to do this, we investigated both the serotonergic and dopaminergic system in individually characterized feather pecking phenotypes (**Chapter 2**) and compared lines genetically selected for low mortality due to injurious pecking (LML) vs. controls (CL) (**Chapter 3**) and for low (LFP) vs. high feather pecking (HFP) (**Chapter 4 and 6**). In the same birds we observed behavior during the rearing phase and/or during the laying phase and sampled brain tissue afterwards (**Chapter 2, 3, and 4**). For the first time ever a procedure to perform *in vivo* microdialysis in adult hens was successfully developed (**Chapter 5**). A microdialysis study was done in freely moving adult laying hens to investigate the monoamine release in a brain area involved in emotional regulation and motor output in individuals from the LFP vs. HFP lines (**Chapter 6**). In this final Chapter, I discuss the most important findings and also postulate a new hypothesis on SFP and the role of monoamines in its development. Furthermore, I give recommendations for future neurobiological research in chickens and describe the possible impact of this type of research in practice.

SEROTONERGIC AND DOPAMINERGIC SYSTEMS DIFFER WITHIN PHENOTYPES AND GENOTYPES DIVERGING IN THE DEGREE OF INJURIOUS PECKING BEHAVIOR

As described in **Chapter 2** a comparison was made between **phenotypically** characterized adult chickens that differed in the degree of feather pecking. Behavioral observations during the adolescent phase (19 to 21 weeks of age) identified severe feather pecking chickens (named SFPs), victims of SFP (only receiving and not giving SFP) and non-peckers (named NPs; not receiving nor giving SFP). Brain tissue samples were collected from four brain areas and it was found that chickens characterized as SFPs had higher serotoninergic activity (depicted by higher metabolite levels or higher turnover ratios) compared to victims and NPs in the dorsal thalamus and medial striatum. Interestingly, in the dorsal thalamus both SFPs and victims had higher 5-HT turnover than NPs and a relation was suggested between the alterations in the serotonergic system and the possible stress associated with SFP, that is, both in the actors and the victims. This assumption was based on previous studies suggesting feather peckers having an altered stress-response (van Hierden et al., 2002; Korte et al., 1997; Vestergaard et al., 1997; Kjaer and Guemene, 2009), but also victims might perceive SFP as unpredictable and stressful. Victims showed altered

serotonergic-related concentrations in the thalamus and striatum similar to rats subjected to unpredictable tail-shocks (Adell et al., 1988). Apart from potential contributions of (long-term) stress to alterations in brain monoamine systems, the higher serotonergic turnover in the SFPs might also be explained by their genotype. Several genetic studies have linked polymorphisms in genes related to both the serotonergic and dopaminergic system to the expression of SFP behavior (Biscarini et al., 2010; Uitdehaag et al., 2011; Labouriau et al., 2009; Wysocki et al., 2010; Wysocki et al., 2013; Flisikowski et al., 2009). Previously, it has been shown that SFP is moderately heritable (Rodenburg and Koene, 2003; Kjaer and Sørensen, 1997) and it is therefore suggested that selection on SFP behavior might directly affect the monoaminergic system. To investigate this further, in **Chapter 3** and **Chapter 4** adult chickens of two lines genotypically selected on traits related to SFP were compared. In **Chapter 3**, hens indirectly selected on low SFP, that is, selected on low mortality due to injurious pecking (LML) were compared with a control line (CL), and in **Chapter 4** two lines divergently selected on High and Low severe Feather Pecking (HFP vs. LFP) were compared by a similar brain tissue sample-methods as used in **Chapter 2**. **Table 7.1** gives an overview of the monoaminergic levels and/or turnover ratios measured throughout this thesis of hens with SFP-prone phenotypes or genotypes.

With the so-called 'punch'-method, it was revealed that selection on SFP affected both the serotonergic and dopaminergic systems in adult hens. It was found that genetic lines high in SFP (CL and HFP) had higher 5-HIAA levels in the arcopallium (**Chapter 3** (tendency) and **Chapter 4**) and higher 5-HT turnover in the NCC (**Chapter 4**) compared to the lines low in SFP (LML and LFP). Although differences between phenotypically selected SFPs and non-peckers were found in other areas than the areas affected by genetic selection, SFP in adult laying hens seems generally related to a higher metabolic activity of the serotonergic system depicted by either higher metabolite levels or higher 5-HT turnover ratios. For dopamine, similar to higher HVA in the arcopallium of phenotypically characterized SFPs (**Chapter 2**), **Chapter 3** and **Chapter 4** revealed higher concentrations of the other DA-metabolite, DOPAC, in the lines high (CL and HFP) compared to the lines low in injurious pecking (LML and LFP) in this same brain area. This also fits with higher DOPAC levels in the rostral part of the brain of adult chickens from a White Leghorn line with more feather damage due to FP in comparison with a Rhode Island Red line low in feather damage (Uitdehaag et al., 2011). The NCC, however, showed lower DOPAC and DA in HFP hens, although HFP hens had a higher DA turnover ratio compared to LFP hens in this area (**Chapter 4**). Both the arcopallium and NCC stand out in these adult hens of the genetic selection lines. The NCC is a large associative frontal area in chickens and predominantly limbic (Shanahan et al., 2013; Atoji and Wild, 2009).

Table 7.1. Overview of monoaminergic levels in young and adult chickens with high propensity to develop injurious pecking in several brain areas.

Technique	Brain area	Serotonin		Dopamine	
		Young[1] (Ch.4)	Adult[2]	Young[1] (Ch. 4)	Adult[2]
Punches	Thalamus	5-HT ↓ 5-HIAA ↓	t.o. ↑ (Ch. 2) 5-HIAA ↑ (Ch. 2) ↔ (Ch. 3)	t.o. ↓	DOPAC ↑ (Ch. 3) ↔ (Ch. 4)
	Medial striatum	t.o. ↓ 5-HIAA ↓	↔ (Ch. 3)	t.o.↓	↔ (Ch. 4)
	Amygdala	5-HIAA ↓	↔ (Ch. 3)	↔	↔ (Ch. 4)
	Arcopallium	t.o. ↓	5-HIAA ↑ (Ch. 4, Ch. 3 trend)	↔	DOPAC ↑ (Ch. 3, 4) HVA ↑ (Ch. 2)
	NCC	t.o. ↓ 5-HIAA ↓	t.o.↑ (Ch. 4) ↔ (Ch. 3)	↔	t.o.↑ (Ch. 4) DA ↓ (Ch. 4) DOPAC ↓ (Ch. 4)
	NCL	↔	↔	↔	↔
	Hippocampus	↔	↔ (Ch. 2)	↔	t.o. ↓ (Ch. 3)
Microdialysis (Ch. 6)	NCC/NCL	X	Baseline: 5-HT ↑ 5-HIAA ↔ post-F: 5-HT ↔ 5-HIAA ↔	X	n.d.

Comparison between chickens with high and low tendency to SFP; victim phenotypes (see **Chapter 2**) not included; ↑ = HFP > LFP, ↓ = HFP < LFP; ↔ = no differences between phenotypes or genotypes; X = no microdialysis performed in young chicks; n.d. = not detected, t.o. = turnover

[1] Results based on monoaminergic levels genetic lines selected for high SFP (HFP) compared to low SFP (LFP) at a young age as presented in **Chapter 4**.

[2] Results based on monoaminergic levels in phenotypically high SFPs compared to non-peckers (**Chapter 2**); in hens from a genetic control line (CL) compared to a line selected for low mortality due to injurious pecking (LML), **Chapter 3)** and in HFP vs. LFP (**Chapter 4 and 6**).

The arcopallium processes somatosensory information (Herold et al., 2011a; Durstewitz et al., 1998; Durstewitz et al., 1999a; Herold et al., 2012b) and receives input from the nidopallium (Metzger et al., 1998; Kröner and Güntürkün, 1999; Atoji and Wild, 2009). Both regions contain serotonergic and dopaminergic afferents (Durstewitz et al., 1998) and receptors (Durstewitz et al., 1999b; Herold et al., 2012b; Herold et al., 2011b). It is suggested that deficits in the monoaminergic system in these brain areas have the potential to affect both the emotional perception and behavioral output in chickens (see also **Chapter 4)**.

In summary, similar to what was found in the phenotypically selected SPF chickens (**Chapter 2**), adult hens from genetic lines displaying high injurious pecking seem to be characterized by higher serotonergic activity (i.e. higher 5-HIAA levels or

higher 5-HIAA/5-HT ratios). Furthermore, **Chapter 3** and **Chapter 4** provide evidence for genetic selection, either directly or indirectly on SFP, affecting the serotonergic and dopaminergic system in adult chickens. The question can be posed whether these differences are already visible in young chickens. After all, previous literature related low serotonergic activity in young chickens to high feather pecking (van Hierden et al., 2002), which is the opposite of our findings in adult laying hens.

MONOAMINERGIC SYSTEMS IN HIGH AND LOW FEATHER PECKERS AT YOUNG AND ADULT AGE

As expected, the monoaminergic systems in young chicks originating from the HFP and LFP lines already differed at a young age, i.e. 8 weeks of age when brain punches were taken. As presented in **Chapter 4** and here in **Table 7.1**, serotonergic activity (mainly 5-HIAA and 5-HT turnover) was lower in young HFP chicks compared to LFP chicks in five of the seven brain areas measured, being the dorsal thalamus, medial striatum, amygdala, NCC, and arcopallium. Dopaminergic differences were less prominent, but were all in the same direction as the serotonergic differences as young HFP had lower DA turnover than LFP in the dorsal thalamus and medial striatum. This fits results of a previous study on the whole rostral brain in young chickens selected on productivity but also divergently differing in feather pecking behavior (van Hierden et al. 2002) and these findings indicate that, low 5-HT and DA turnover ratios at a young age are associated with high feather pecking. Notably, differences in the serotonergic system seem present throughout the brain (with exception of the NCL and hippocampus) at both ages, regardless of the brain area sampled. For DA, however, it depends on the specific brain area whether a higher or lower metabolic activity is found in severe feather pecking birds. This difference may be due to the different anatomy of these neurotransmitter systems: most brain areas are innervated by serotonergic fibers, therefore it is generally accepted that the specificity of the serotonergic system comes from the many (14 post-synaptic) 5-HT receptors (Herold et al., 2012b; Hoyer et al., 2002). In contrast, the variety of DA-receptors is less broad (Kubikova et al., 2010) and specificity of the dopaminergic system seems therefore to depend particularly on dopaminergic fiber projections to specific target areas with certain areas more highly innervated than others (Durstewitz et al., 1999b; Schnabel et al., 1997). For instance, the medial striatum is highly innervated with dopaminergic fibers and 10-fold higher DA-concentrations were measured in this area than in surrounding brain areas.

Interestingly, when comparing these neurochemical results with those measured in adults, it becomes apparent that the pattern of the relation between monoamines and feather pecking is reversed. **Table 7.1** summarizes the findings in both young and adult chickens used in this thesis and shows that whereas young HFP were characterized by lower neurochemical 5-HT and DA levels than LFP, adult hens with a high propensity for SFP had higher levels compared to the low SFP hens. In contrast, HFP chickens displayed an overall constant higher level of behavioral activity

in comparison to LFP chickens over the course of the study described in **Chapter 4**. Young HFP showed more allopecking, i.e. more SFP and toe pecking although equally high frequencies of gentle pecking were recorded between lines, and HFP displayed more active behavior in the behavioral tests conducted. This displayed behavior fits in the profile of individuals with a hyperactivity disorder, as already suggested by Kjaer (2009). At the neurobiological level, the young HFP are characterized by lowered 5-HT turnover and DA turnover in brain regions related to the frontal-thalamic-striatal circuit with deficits in this circuit leading to an increased risk to develop disorders related to (hyper)activity, impulsivity and compulsivity in mammals (Angoa-Pérez et al., 2012; van den Heuvel et al., 2010; Chamberlain et al., 2009; Fineberg et al., 2010; Insel et al., 1985; Pattij and Vanderschuren, 2008; Bechara et al., 1994; Chamberlain and Sahakian, 2007). The actual cause of the inversion of neurochemical patterns in young and adult LFP and HFP birds remains to be elucidated. It is unknown whether this inversion just occurs in these genetic lines over the course of development, or whether the divergent behavioral patterns in these chickens may in some way have influenced brain monoaminergic activity. A recent line of research hypothesizes that the serotonergic system is influenced by ingestion, and thereby produces higher locomotor activity as observed in SFP birds. That is, SFP has been related to feather eating (McKeegan and Savory, 1999). In addition, HFP birds have been shown to differ from LFP chickens in intestinal microbial metabolites (Meyer et al., 2013). Feather eating has effects on feed passage rate in the gastrointestinal tract (Harlander-Matauschek et al., 2006), affects gut mircobiota and microbial metabolites (Meyer et al., 2013). Interestingly, chopped feathers added to the diet of laying hens have been reported to reduce SFP and improve plumage condition (Kriegseis et al., 2012). Indeed, birds from the HFP line seem more motivated to work for feathers and have a higher preference for feathers as a substrate than LFP birds (Harlander-Matauschek et al., 2006; Harlander-Matauschek et al., 2007). This suggests that feather eating may affect intestinal functioning and behavior, i.e. execution of this feather pecking and feather eating behavior may have functional consequences, at least at the gut level, and, given the behavioral effects of feather eating, perhaps also at the brain level. Recently, several studies showed an important influence of gut-microbiota on brain functioning and neurotransmitter systems (Stilling et al., 2013). All data gathered so far indicates a role for the gut-brain axis in FP. Interestingly, 5-HT in the intestines is involved in gut peristaltic movements and LML birds have higher whole blood 5-HT levels (is mainly storage of 5-HT derived from the intestine in platelets) (Rodenburg et al., 2009; Bolhuis et al., 2009). These intracellular 5-HT levels might have been influenced by possibly altered serotonin transporter (SERT) activity (Buitenhuis et al., 2006). A study on the relation between peripheral and brain 5-HT in pigs showed that tail biting pigs have lower blood platelet 5-HT levels too, in those phases during which tail biting was observed (Ursinus et al., 2013). Although the above opposed explanation for how neurochemical deficits might be diminished by chickens adjusting their behavior (e.g. by eating feathers), feather peckers seem very persistent in their

behavior since SFP incidences were still very high in adult HFP birds. These birds even displayed aggressive behavior from the age of 13 weeks onwards. Again, this might be caused by lowered 5-HT turnover early in the development, since administration of 5-HT in the egg lowered aggressiveness measured in adolescent chickens (Dennis et al., 2013a) which might explain the young LFP to display little of this hierarchical behavior (as they have higher 5-HT at a young age). Or, the activated dopaminergic system when adult has a part in this persistent behavior. Whatever the reason, some caution has to be attributed with interpreting these neurochemical results and its relation with SFP behavior. That is, although studying more specific brain areas, as presented in this thesis, provided more detailed information on the role of 5-HT and DA in SFP than by analyzing the whole rostral brain (van Hierden et al., 2002; Uitdehaag et al., 2011) or larger brain areas (van Hierden et al., 2004), by using the punch-method no direct information is gathered on the release of neurotransmitters. Importantly, the release of monoamines is biologically relevant for the output of behavior and microdialysis is the suitable technique for measuring this.

MICRODIALYSIS IN ADULT LAYING HENS WITH A PROPENSITY FOR HIGH AND LOW FEATHER PECKING

In comparison to the punch-method, microdialysis enables collecting multiple samples over a period of time containing neurotransmitters, such as 5-HT and DA released in the synaptic cleft in a certain brain area, while the animal is able to freely move. As described in **Chapter 5**, microdialysis in chickens is not very common: only 19 studies refer to microdialysis in chickens compared to over 8.300 in rats (via a PubMed search). These 19 studies only refer to microdialysis in young chickens, up to 25 days of age, and concern topics like imprinting and feeding (e.g. (Tachibana et al., 2000; Ichijo et al., 2008; Gruss and Braun, 1996b), but not feather pecking. Given that SFP in laying hens usually develops later in life - at the onset of lay (Uitdehaag et al., 2011; Gilani et al., 2013; Newberry et al., 2007), and considering our findings of adult HFP differing from LFP in their serotonergic and dopaminergic activity (**Chapter 4**), microdialysis in adult hens can provide additional information on monoamines in relation to SFP. **Chapter 5** described the protocol used to successfully perform microdialysis in adult chickens. Compared to the protocol used in our lab for microdialysis in rats and mice (Prins et al., 2011a; van Heesch et al., 2013; van der Stelt et al., 2005), the most obvious adjustments were 1) the raised stereotaxic apparatus with a chicken adaptor; 2) the protection of the probe with a metal casing and securing tape; and 3) the anesthesia via gas inhalation that enabled precise controlling of the duration of sedation. With these careful adjustments and care for the animals, the new protocol proved successful for measuring monoamines in the extracellular fluid. **Chapter 6** described the results of the microdialysis performed in laying hens originating from HFP and LFP lines. The microdialysis probe was aimed at the nidopallium. This was for three reasons: Firstly, **Chapters 3 and Chapter 4** revealed that both serotonergic and dopaminergic differences between adult hens of

the HFP and LFP line were most evident in the caudocentral part of the nidopallium (NCC). Secondly, monoaminergic differences were also present in the arcopallium (intermedium), an area with reciprocal projections to both the caudocentral nidopallium (NCC) and the caudolateral nidopallium (NCL) (Metzger et al., 1998; Kröner and Güntürkün, 1999; Atoji and Wild, 2009). Thirdly, the nidopallium is a large frontal brain area within relative easy reach of a probe. This was important since determining the exact coordinates of the probe was complicated by the absence of a brain atlas for adult chickens. An extensive pilot study (shortly described in **Chapter 5** and **Chapter 6**) was therefore necessary to determine the coordinates of the nidopallium.

In vivo microdialysis revealed that chickens with a high tendency to perform SFP (HFP hens) had a higher baseline release of 5-HT compared to the hens with low tendency to perform SFP (LFP hens). These higher levels in HFP could not be explained by the amount of 5-HT presynaptically stored, as both lines displayed a similar 5-HT release after d-fenfluramine administration. Also, levels of the 5-HT precursor tryptophan in blood plasma were similar between lines. Therefore, these results suggest that divergent genetic selection on SFP alters the serotonergic system in such a way that HFP laying hens have a higher tonic baseline release of 5-HT. Interestingly, the higher 5-HT release in HFP might also be closely related with the overall higher behavioral activity displayed by these animals in different behavioral tests as described in **Chapter 4** and in other studies (Kjaer, 2009; de Haas et al., 2010), although it cannot be excluded that also DA plays an important role considering the dopaminergic effects found in the arcopallium (**Chapter 3**). According to Jacobs and colleagues (Jacobs et al., 2002; Jacobs and Fornal, 1997; Jacobs and Fornal, 1993) 5-HT has a distinct role in motor function. Their work on the serotonergic system in cats clearly shows that the level of tonic motor activity is positively related to 5-HT neuronal activity across all groups of 5-HT neurons (Jacobs and Fornal, 1999). They further hypothesized that serotonergic neurons are activated in response to repetitive behaviors. Thus, execution of behavior itself can lead to changes in the brain as well as changes in the brain can affect behavior. Following this hypothesis, Jacobs and Fornal (1997) suggested that the behavioral repetitiveness in patients with obsessive-compulsive disorder is a mean to activate their (impaired) serotonergic system in a biological manner. In line with this trace of thought, it is highly speculative whether the lower serotonergic activity (lower 5-HT turnover) in young HFP chickens in **Chapter 4** has caused these same animals to increase their motor activity; quite successfully, if you would believe this has affected the later recorded higher serotonergic activity (higher 5-HT turnover) in the punched samples (**Chapter 4**) and the increased 5-HT neurotransmission via microdialysis (**Chapter 6**) in the adult HFP chickens. Nonetheless, this microdialysis study should be repeated in young chickens of the HFP and LFP line to better discriminate between the cause and consequence of altered serotonergic system activity in relation to the behavioral output.

THE GENOTYPE-PHENOTYPE MODEL OF SEVERE FEATHER PECKING

My thesis emphasizes the importance of *phenotypic discrimination* in chickens since severe feather peckers differ from victims and the non-peckers in the activity of the central serotonergic system (**Chapter 2**). Also genetic selection on (traits related to) SFP has caused differences in the serotonergic and dopaminergic system between selection lines (**Chapter 3, Chapter 4, Chapter 6**). The question: "Why do laying hens develop SFP behavior?" keeps scientists searching for solutions already for more than 40 years (Hughes, 1973). Even today, SFP behavior is still a huge animal welfare problem. Here (and in **Chapter 2**), I propose a new model to explain the development of SFP based on the existence of 1st and 2nd order feather peckers. This model offers new opportunities for future research on brain-behavior relations regarding SFP. **Fig 7.1** illustrates this model, called 'the genotype-phenotype model of severe feather pecking'.

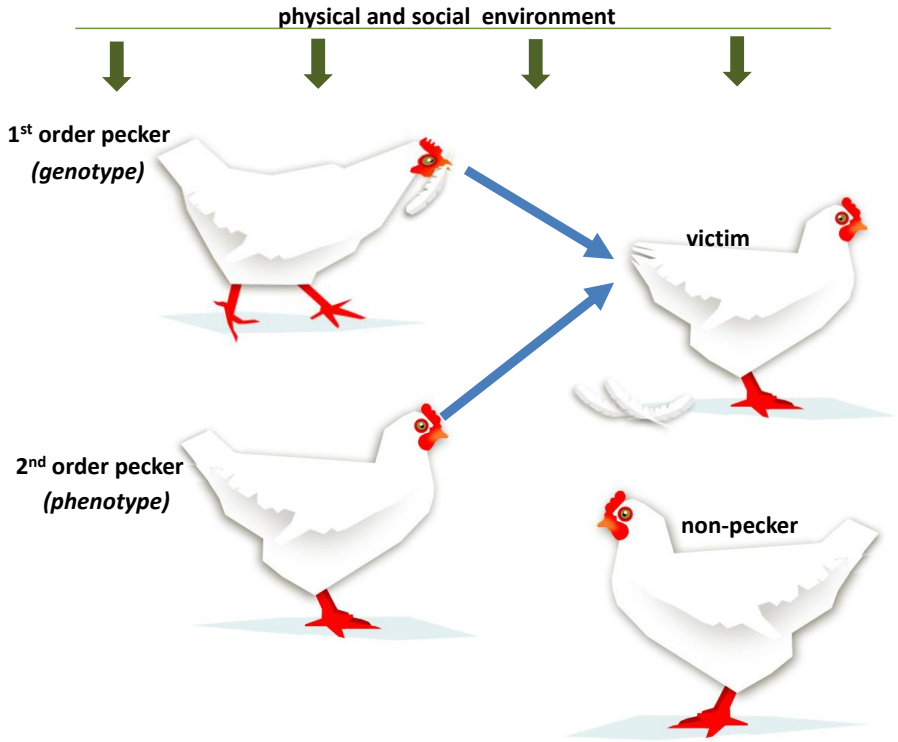

Fig. 7.1 The genotype-phenotype model of severe feather pecking. This model assumes the existence of different phenotypes of severe feather peckers: '*First order feather peckers'* are genetically predisposed (intrinsic factors) to develop SFP under pressure from environmental stressors such as husbandry conditions (physical stressors) and group size and density (social stressors). '*Second order feather peckers'* start SFP if their attention is drawn to the damaged feathers of the victim (external factors) without any neural "deficits" underlying this behavior.

Evidence for this model comes partly from genetic studies demonstrating a moderate heritability of SFP (Su et al., 2005; Rodenburg and Koene, 2003; Kjaer and Sørensen, 1997; Bessei, 1984). Remarkably, several genetic and pharmacological studies further established the involvement of both central 5-HT and DA in FP (van Hierden et al., 2004; Bordnick et al., 1994; Kjaer et al., 2004; van Hierden et al., 2004; van Hierden et al., 2005; Biscarini et al., 2010; Flisikowski et al., 2009). Therefore, it is concluded that certain chickens may have a genetic predisposition to develop injurious pecking. In **Chapter 4**, I showed that SFP is much more widespread among lines selected on high severe feather pecking (HFP) than lines selected on low feather pecking (LFP) (see **Table 4.2)**.

Importantly, only a few individuals within a group usually start with SFP (Rodenburg et al., 2013). This indicates that not only the chickens' genotype determines whether individuals will start pecking at and pulling out feathers, but also environmental factors play a role (Rodenburg et al., 2008; McAdie et al., 2005).

External factors such as housing conditions, feeding, lighting, group size, and density directly affect SFP behavior (Rodenburg et al., 2008; Johnsen et al., 1998; Kjaer and Sørensen, 2002). The presented model assumes that the impact of above-mentioned environmental factors is largest in these genetically predisposed birds. These individuals are the '*1st order feather peckers'*. It is hypothesized that their ability to cope with altered environmental situations is hampered by their genes. Several behavioral tests, including the ones in this thesis, have shown that HFP birds are more active, also during challenging tests (Kjaer et al., 2001; de Haas et al., 2010), indicating that feather pecking may be genetically linked to a proactive coping strategy (reviews by (Koolhaas et al., 1999; Korte et al., 2009); thereby explaining individual variances in the development of feather pecking. It is known that severe feather pecking increases over time; with a high risk of SFP outbreaks occurring around the time chickens start laying eggs, suggesting the involvement of gonadal hormones, especially the sex steroids estrogen and progesterone (Hughes, 1973). However, in commercial practice, adolescent chickens (around age 16-20 weeks) are transferred from rearing farms to egg laying farms, and this change of environment might induce feather pecking as well.

Nevertheless, the number of chickens displaying this injurious pecking behavior increases over time as seen in **Table 4.2** (from 39 % in young to 55% when adolescent). Possibly, SFP can be transmitted to other birds. In **Chapter 2**, I proposed to call these 'new' feather peckers the '*2nd order feather peckers'*. In contrast to the 1st order peckers which in response to mostly endogenous stimuli show SFP, the 2nd order peckers mainly respond to external stimuli. Only under the influence of both physical and social stressors and other external stimuli such as ruffled feathers and bold spots on victim, these 2nd order peckers may start damaging the feathers of group members. Besides the above mentioned changes in physical and social environment around the onset of lay – affecting all chickens – also the presence of SFP birds may increase stress hormone levels, such as corticosterone concentrations in the blood at group

level (de Haas et al., 2012). It has recently been shown that the presence of fearful group members may increase fear and stress responses in their group members too (De Haas et al. 2012), which, in turn, given the association between fear, stress and SFP (Rodenburg et al., 2004) may increase the risk of feather pecking. The previously non-pecking chickens (giving gentle pecks, but not giving or receiving severe pecks) may also increase the severe pecking at feathers by a process called social transmission (Zeltner et al., 2000; Nicol, 1995), and/or might respond to the appearance of damaged or ruffled feathers (McAdie and Keeling, 2000). Previously, it has been suggested that animals adopting a reactive (passive) coping style respond more to subtle environmental changes (Koolhaas et al., 1999). Therefore, the 2nd order would fit the profile of passive copers and, hence, better notice feather damage. Importantly, the hypothetical presence of both 1st and 2nd order feather peckers within a group might explain the sometimes contrasting results on the behavioral and physiological phenotype of (suggested) feather peckers. For instance, chickens prone to develop severe feather pecking have been suggested to be more fearful (Rodenburg et al., 2009; de Haas et al., 2012; Hocking et al., 2001; Bolhuis et al., 2009; Nordquist et al., 2011), but also highly impulsive (Kjaer et al., 2001; this thesis).

In **Fig. 7.2** (see pg. 122), the effect of time and development is integrated in the "genotype-phenotype model of severe feather pecking". It shows how 1st order peckers develop under the influence of both genetic (G_1) and environmental (E_1) factors, resulting in a changed environment (E_2) with victims (with damaged feathers) and non-peckers. In this context, 2nd order peckers can evolve. This larger group of both 1st and 2nd order peckers will lead to more victims, increased fear (both at group and individual level) and feather damage. Although gonadal hormones are not studied in my thesis, it can be hypothesized that sexual maturation, a process during which sex steroids directly affect gene expression, result in even more damaging pecking behaviors such as vent pecking, which can easily progress into cannibalism ($E_2 \rightarrow E_3$). Therefore, in the future the influence of gonadal hormones on gene expression, which has been shown to be involved in brain monoaminergic neurons (Reisert and Pilgrim, 1991) and SFP (Hughes and Duncan, 1972), needs further investigation in the new model presented here. This may open up new possibilities for solving the difficult animal welfare problem of SFP.

FUTURE PERSEPECTIVES AND LIMITATIONS

With differences found in the activity of the central monoaminergic system between high and low feather pecking birds, I feel my thesis justifies future neurobiological research aimed at clarifying the cause and consequence of SFP behavior in relation to brain monoamines, especially trait differences. Since microdialysis in adult chickens is now made possible; this opens new possibilities to study the underlying role of neurotransmitters in SFP behavior more directly. By combining microdialysis in severe feather pecking laying hens treated with pharmaceuticals such as selective

monoamine reuptake inhibitors, specific receptor agonists and receptor antagonists, fundamental questions regarding the neural mechanism of feather pecking behavior and the neurophysiological characteristics of chickens prone to develop SFP, can now be addressed. For instance, a remaining question is whether serotonin transporter (SERT) functioning differs between high and low feather peckers, given that both phenotypically and genetically selected adult SFP chickens seem characterized by higher serotonergic activity, as depicted from both the microdialysis and brain punch analyses. That is, it can be speculated that the act of SFP or feather eating as such has direct effects on brain monoamine activity, and serves as a compensatory mechanism to alleviate brain monoamine deficiencies. Adding to this, it would be interesting to investigate the role of gonadal hormones.

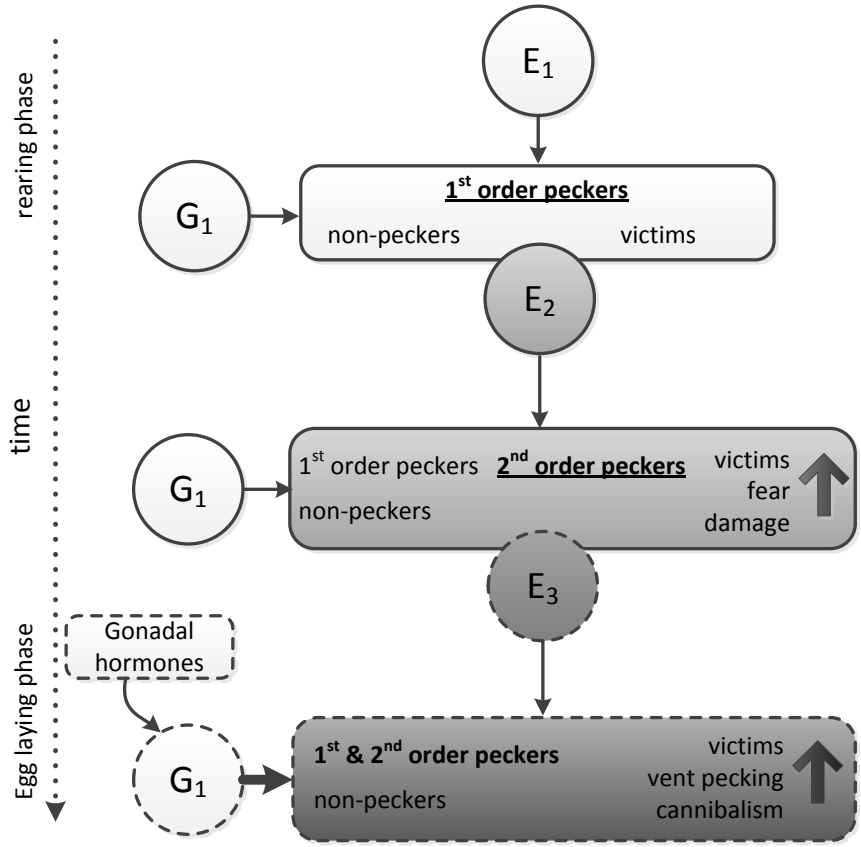

Fig. 7.2 Integrating the effects of time and development in the genotype-phenotype model of severe feather pecking. First order peckers develop under the influence of both genetic (G_1) and environmental (E_1) factors, resulting in a changed environment (E_2) with victims (with damaged feathers) and non-peckers. In this context, second order peckers can evolve. This larger group of both 1st and 2nd order peckers will lead to more victims, increased fear (both at group and individual level), and increased feather damage. During sexual maturation, it is hypothesized that gonadal hormones change the activity of certain neural networks, resulting in even more damaging pecking behaviors such as vent pecking, which can easily progress into cannibalism.

Increased testosterone levels – indicative for maternal effects – can result in brain lateralization and affected distribution of androgen receptor (e.g. Pfannkuche et al., 2011; Pfannkuche et al., 2009), thus potentially affects brain circuits involved in SFP. Linking neurochemical deficits directly to SFP is challenging, however, although currently promising progress is made in our lab: we are investigating ways to accurately measure very low quantities of dialysate in order to shorten the time-frame of microdialysis sampling to 1-2 minutes (instead of the standard 30 min.). If successful, this will create opportunities to measure released monoamines while at the same time bouts of pecking are observed.

In addition, with the current knowledge, it seems valuable besides comparing genetic lines diverging in SFP, to make phenotype-within-genotype comparisons at different ages. This will help to further disentangle the neurochemical causes and consequences of feather pecking. Identifying 1st and 2nd order peckers asks for careful behavioral observations and testing. More studies have to be performed to discriminate between the possible underlying motivations (e.g. low social motivation, high impulsivity and/or low fear) of severe feather peckers, because their general response pattern appeared to be characterized by fast responding and high locomotor activity levels. Therefore, behavioral paradigms of choice should offer a read-out that does not solely depend on activity levels, and, preferably, allow for a multidimensional characterization of feather peckers (e.g. coping style *and* fearfulness or stress sensitivity). Although time consuming, characterization of the personality of soon-to-be (1st order) peckers can be helpful in interpreting brain results. Rather simple behavioral paradigms can also be more easily applied in practice, on poultry farms. Another applicable approach is to take blood samples. Differences in the peripheral serotoninergic and dopaminergic system have been found in lines selected on high and low survivability (mortality caused by FP and cannibalism) (Rodenburg et al., 2009; Cheng and Muir, 2007; Bolhuis et al., 2009; Cheng et al., 2001a; Buitenhuis et al., 2006) and the predictive validity of peripheral levels with respect to central (brain) levels (Uitdehaag et al., 2011; Bianchi et al., 2002) should be further investigated.

CONCLUDING REMARKS

The 'genotype-phenotype model of severe feather pecking' introduced in this thesis is based on fundamental brain research in combination with behavioral research, and this model stresses the importance of considering the impact of genetic selection and environmental conditions – important issues for breeders and farmers – on brain chemicals and with that, on the vulnerability of individual chickens to develop SFP. With SFP being a multifactorial problem, there is not one answer for it, but by taking genotype and phenotype in consideration, brain research is valuable for addressing questions that go beyond fundamental research.


Afferent and Efferent Projections of the Central
Nidopallium in the Pigeon (Columba livia)
YASURO ATOJI AND J. MARTIN WILD
Organization of the Dopaminergic
Innervation of Forebrain Areas
Relevant to Learning: A Combined
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Study in the Domestic Chick
The dorsocaudal ne
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Neuroscience Letters
Expression of the neocortical marker, RORβ, in the entopallium and field L2 of
adult chicken
Yasuro Atoji
PERSPECTIVES
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Revised Nomenclature for Avian
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Samenvatting in het Nederlands

ERNSTIG VERENPIKKEN, EEN PROBLEEM IN LEGHENNEN

Ernstig verenpikken veroorzaakt welzijnsproblemen in leghennen. Doordat hennen pikken naar veren en deze uittrekken ontstaat er schade aan het verenkleed en de huid van hun slachtoffers. De ontstane veerschade en kale plekken leiden niet alleen tot een verminderd welzijn bij de slachtoffers, maar verhogen ook het risico om voortdurend gepikt te worden, met uiteindelijk zelfs kannibalisme en de dood van slachtoffers tot gevolg.

Externe factoren zoals huisvesting, lichtintensiteit, groepsgrootte en opfokcondities hebben een aantoonbare invloed op de ontwikkeling van verenpikken (zie ook **hoofdstuk 1**). Wat het voorspellen van dit gedrag lastig maakt is dat ernstig verenpikken zich vaak pas op latere leeftijd ontwikkelt met vaak bepaalde individuen in de groep als voorloper. Genetische factoren spelen hierbij een rol (verenpikken blijkt deels overerfbaar), maar er zijn ook duidelijk aanwijzingen dat neurotransmitters (boodschapperstoffen in het zenuwstelsel), zoals de monoamines serotonine (5-hydroxytryptamine; 5-HT) en dopamine (DA) een aandeel hebben in de gevoeligheid voor het ontwikkelen van verenpikgedrag. Eerder onderzoek in jonge kuikens heeft aangetoond dat lage gehaltes van deze neurotransmitters in de hersenen het risico op verenpikken vergroten (van Hierden et al. 2002). Serotonine staat vooral bekend om zijn invloed op de regulatie van de gemoedstoestand, voortplanting, groei, maar ook impulsiviteit en agressie; en dopamine om zijn rol in motivatie en beloning, maar ook motorische controle. Uit zowel humaan onderzoek als onderzoek bij proefdieren is bekend dat afwijkende gehaltes van 5-HT en DA het risico vergroten op psychopathologieën en gedragsstoornissen, zoals angststoornissen en impulsiviteits- en compulsieve stoornissen. Door beter te begrijpen welke oorzakelijke factoren ten grondslag liggen aan het verenpikgedrag, kan beter behandeld en mogelijk voorkomen worden.

Het doel van dit proefschrift was inzicht te verkrijgen in de hersenmechanismen onderliggend aan het ernstig verenpikgedrag bij leghennen met speciale aandacht voor serotonine en dopamine.

De studies in dit proefschrift richten zich op de volgende vragen: Wat is het effect van fenotype en genotype met betrekking tot verenpikken op het serotonerge en dopaminerge systeem? Verschillen jonge en volwassen hennen van divergente verenpiklijnen (hoog versus laag verenpikken) in de neurotransmitterwerking van verschillende hersengebieden? Ten slotte, is het mogelijk om neurotransmitters tussen de zenuwcellen (synaptische spleet) te meten in vrij rondlopende volwassen kippen met behulp van *in vivo* microdialyse en zo ja, verschillen verenpikkers van niet-verenpikkers in de neurotransmitter-afgifte in relevante hersengebieden?

Gebruikte methodes

Bij hersenonderzoek is de samenhang tussen hersenen en gedrag een belangrijk gegeven. Voor de interpretatie van 'hersendata' is het cruciaal te weten welk gedrag het dier heeft vertoond of waartoe het (genetisch) geneigd is. In dit proefschrift wordt onderscheid gemaakt tussen *genotypische* verenpikkers (geselecteerd op erfelijke kenmerken gerelateerd aan verenpikken via fokprogramma's) en *fenotypische* verenpikkers (selectie op basis van het vertoonde verenpikgedrag). Gedragsobservaties en gedragstesten zijn op jonge en volwassen leeftijd verricht met als doel de kippen te karakteriseren. Twee neurologische technieken zijn in dit proefschrift gebruikt: 1) het verzamelen van hersenmateriaal uit hersencoupes, genaamd 'punches' en 2) *in vivo* microdialyse. *In vivo* microdialyse is een techniek waarbij de neuronale afgifte van neurotransmitters zoals monoamines direct gemeten kan worden in levende dieren. Waar de eerste methode gehaltes van neurotransmitters in verschillende hersengebieden tegelijkertijd kan meten, is de tweede methode nauwkeurig in het meten van de neurotransmitter afgifte in één enkel gebied. Beide methodes bepalen concentraties van 5-HT en DA en hun metabolieten (afbraakproduct) en uit de punch-methode kan ook de turnover ratio berekend worden. Het afbraakproduct van 5-HT is 5-HIAA (5-hydroxyindole acetic acid) en DA wordt afgebroken tot DOPAC (dihydroxyphenyl acetic acid), HVA (homovanillic acid) en 3-MT (3-methoxytyramine). Turnover is een maat voor neuronale activiteit en is de verhouding tussen de hoeveelheid metaboliet ten opzichte van de hoeveelheid monoamine in het systeem. Een hoger getal geeft een hogere omzetting (verbruik) van monoamines aan.

SEROTONERGE EN DOPAMINERGE VERSCHILLEN TUSSEN FENOTYPES EN GENOTYPES DIE DIVERGEREN IN DE MATE VAN PIKKEN

Fenotypes

Verenpikgedrag is veelal op groepsniveau bestudeerd. Toch moet het probleem ook vanuit het individu bekeken worden, aangezien vaak maar enkele kippen met het verenpikgedrag beginnen. Daarom onderscheidden we in **hoofdstuk 2** verenpikkers van hun slachtoffers en van niet-pikkers.

Volwassen verenpikkers bleken van slachtoffers en van niet-pikkers te verschillen in hun serotonerge activiteit in de hersenen. Analyse van vier hersengebieden liet zien dat volwassen verenpikkers, in vergelijking tot niet-pikkers, hogere 5-HIAA gehaltes hadden in de dorsale thalamus en een daaraan gerelateerde hogere 5-HT turnover (5-HIAA/5-HT) (zie **Fig. 2.1**). De waardes voor de slachtoffers lagen daar tussenin. Het mediale striatum gaf ook serotonerge verschillen; de niet-pikkers hadden hogere 5-HT en de verenpikkers hogere 5-HIAA gehaltes ten opzichte van de slachtoffers. Het arcopallium en hippocampus lieten geen fenotypische verschillen zien. Ook werden

nergens effecten op DA-turnover waargenomen. Wel hadden verenpikkers meer HVA dan niet-pikkers en was er een trend voor hogere HVA in slachtoffers versus niet-pikkers.

De thalamus en het striatum zijn gebieden betrokken bij het reguleren van motorische acties en besluitvorming door het integreren van sensorisch-motorische, motivationele en emotionele informatie. Het is bekend dat afwijkingen in neuronale activiteit de gevoeligheid voor het ontwikkelen van verstoord gedrag, zoals een angststoornis, een impulsieve stoornis of een compulsieve stoornis bevorderen. Meestal wordt verstoord gedrag echter gerelateerd aan lagere 5-HT en DA gehaltes, terwijl in **hoofdstuk 2** de verenpikkers juist hogere gehaltes hadden. Het is ook opvallend dat, in de thalamus, zowel verenpikkers als slachtoffers hogere 5-HT activiteit hadden in vergelijking tot niet-pikkers. Dit suggereert dat verenpikken zelf een effect heeft op het serotonerge systeem van de betrokkenen; of je nu dader of slachtoffer bent.

Genotypes

De drie fenotypes uit **hoofdstuk 2**, de verenpikkers, slachtofferes en niet-pikkers, werden gekarakteriseerd via gedragsobservaties tijdens de adolescente fase. Er is ook onderzocht of selectie over een aantal generaties op kenmerken gerelateerd aan verenpikken eveneens een effect heeft op het centrale monoaminerge systeem. De kippen gebruikt in **hoofdstuk 3** waren al vier generaties geselecteerd op lage sterfte en lange overleving in groepen met onbehandelde, intacte snavels (de *'low mortality line', LML*). In zulke groepen leiden verenpikken en kannibalisme tot veel uitval. De LML lijn werd vergeleken met een controlelijn (*CL*). De kippen uit **hoofdstuk 4** waren reeds negen generaties divergent geselecteerd op hun verenpikgedrag wat resulteerde in lijnen met hoog verenpikkers (de *'high feather pecking', HFP*) en laag verenpikkers (de *'low feather pecking', LFP*).

Hersenanalyse liet zien dat selectie op lage sterfte in groepen en op laag verenpikken het serotonerge en dopaminerge systeem van volwassen leghennen beïnvloedt. De genetische lijnen met meer verenpik-incidenten (CL en HFP) hadden hogere 5-HIAA gehaltes in het arcopallium (**hoofdstuk 3**, trend, en **hoofdstuk 4**) en een hogere 5-HT turnover in het caudocentrale nidopallium (NCC, **hoofdstuk 4**) in vergelijking met de lijnen laag in verenpikken (LML en HFP). Gehaltes van DOPAC, een van de DA-metabolieten, was hoger in de genetische hoog verenpikkers (CL en HFP) in vergelijking tot de laag verenpikkers (LML en LFP). Dit is in lijn met de resultaten van **hoofdstuk 2** waarin de fenotypisch gekarakteriseerde verenpikkers hogere gehaltes van HVA, de andere DA metaboliet, hadden in dit hersengebied. In het caudocentrale nidopallium, afgekort tot NCC, hadden HFP echter lagere DOPAC en DA gehaltes, hoewel de DA-turnover (berekend als (DOPAC+HVA)/DA) hoger was in HFP in vergelijking tot LFP.

Verschillen tussen hoog en laag verenpikkers werden vooral gevonden in gebieden betrokken bij de verwerking van emoties en motor-output. Het NCC is een groot gebied in de voorhersenen en geassocieerd met het limbische systeem (verwerking van gevoelens) en heeft bovendien sterke connecties met het arcopallium dat somato-sensorische (zintuiglijke) informatie verwerkt. Beide gebieden werken nauw samen en bevatten zowel serotonerge als dopaminerge neurale uiteindes. Op basis van deze resultaten suggereer ik dat de afwijkingen in het monoaminerge systeem in deze hersengebieden de potentie hebben zowel de emotionele beleving als de gedragsmatige output in kippen te beïnvloeden. Kortom, genetische selectie, direct of indirect, op ernstig verenpikken beïnvloedt het serotonerge en dopaminerge systeem in volwassen leghennen, waarbij de neiging tot verenpikken gerelateerd is aan een algemeen hogere metabolische activiteit van de neurale systemen. Voor verdere interpretatie was het van belang te achterhalen of deze afwijkingen al op jonge leeftijd aanwezig waren, immers, in de literatuur over jonge kuikens wordt juist een lage monoaminerge activiteit gerelateerd aan verenpikken. Daarom is zowel het gedrag als de hersenen van jonge HFP en LFP kuikens geanalyseerd (zie ook **hoofdstuk 4**).

HOOG EN LAAG VERENPIKKERS OP JONGE EN VOLWASSEN LEEFTIJD

Gedrag

Verschillende studies wijzen erop dat verenpikkers angstiger zouden zijn dan niet-pikkers. Dit hebben we onderzocht in de HFP en LFP lijn (zie **hoofdstuk 4**). Wij vonden dat HFP kippen over de gehele studie genomen actiever gedrag vertoonden, zoals het vocaliseren en bewegen (lopen) in bepaalde gedragstesten op jonge leeftijd (in de 'social isolation test' en de 'social runway test'). Dit wordt vaak geïnterpreteerd als het gedrag van niet-angstige dieren. Toch duidt de combinatie van verschillende gedragstesten erop dat de kippen uit de HFP lijn niet zozeer minder angstig zijn dan LFP kippen maar eerder een hogere algemene bewegingsactiviteit hebben. Hun actieve gedrag tijdens twee andere testen (de 'human approach test' en de 'novel object test') was namelijk ook een reflectie van proactief, niet-angstig gedrag. Ook bleek uit verenpikobservaties dat HFP actiever pikgedrag vertoonden dan LFP. Verschillende vormen van pikgedrag zijn geobserveerd, te weten zacht verenpikken, ernstig verenpikken, agressief pikken en teenpikken. Anders dan bij ernstig verenpikken lopen de veren en huid bij zacht verenpikken geen schade op. Agressief pikken richt zich op het hoofd, in tegenstelling tot ernstig verenpikken dat gericht is op de staart-, rug en vleugelveren. Zacht verenpikken wordt gezien als een vorm van exploratief pikken en is waarneembaar vanaf jonge leeftijd. Ernstig verenpikken, daarentegen, ontwikkelt zich vaak pas op latere leeftijd met enkele individuen in de groep als voorloper. Opvallend genoeg blijkt zacht verenpikken op jonge leeftijd niet voorspellend voor ernstig verenpikken op latere leeftijd (Rodenburg et al., 2004;

2013). In de genetisch geselecteerde lijnen bleek zowel zacht als ernstig verenpikken vroeg waarneembaar. Zowel HFP als LFP kuikens vertoonden zacht verenpikken, maar HFP kuikens vertoonden significant meer ernstig verenpikken en teenpikken dan LFP. Bij adolescenten werd naast hogere niveaus van zacht en ernstig verenpikken ook agressief pikken geobserveerd in de HFP lijn.

Hersenen

De bevindingen op hersenniveau geven een mogelijke oorzaak voor het motorisch actievere gedrag van kippen uit de HFP lijn; jonge HFP hadden namelijk een lagere activiteit van het serotonerge en dopaminerge systeem in vergelijking tot jonge LFP. In vijf van de zeven gemeten hersengebieden hadden jonge HFP een lagere serotonerge activiteit (weergegeven met lage 5-HIAA en/of lage 5-HT turnover). Dit werd teruggevonden in de dorsale thalamus, het mediale striatum, de amygdala, het NCC en het arcopallium. De verschillen voor dopamine waren minder prominent, maar wezen wel in dezelfde richting, aangezien jonge HFP een lagere DA turnover hadden dan LFP in de dorsale thalamus en het mediale striatum. Deze bevindingen kloppen met de literatuur en duiden erop dat lage 5-HT en DA turnover ratio's in jonge kuikens geassocieerd kunnen worden met hoog verenpikken op latere leeftijd.

In vergelijking met de bevindingen in de volwassen dieren, geeft dit het interessante beeld dat de relatie tussen verenpikken en monoamines omkeert tijdens de ontwikkeling van jong kuiken naar adolescent. Mogelijkerwijs is het actievere gedrag (inclusief het verenpikgedrag) een compensatie voor de lagere concentraties op jonge leeftijd. De hersenen van kippen ontwikkelen zich namelijk tot in de 10^{e} levensweek en afwijkende concentraties van, met name, serotonine kunnen een langdurig effect hebben op hersencircuits en daarmee ook op het gedrag.

MICRODIALYSE IN VOLWASSEN KIPPEN

Om een preciezer beeld te krijgen van de neurale activiteit is *in vivo* microdialyse uitgevoerd. Microdialyse is een techniek waarmee de afgifte van neurotransmitters over een bepaalde periode gemeten kan worden in een levend (*in vivo*), vrij rondlopend dier. Een probe (een aan- en afvoer buisje met aan het einde een half doorlaatbaar membraan) wordt in een hersengebied gezet en via een gesloten systeem wordt hersenvloeistof opgevangen. De afgifte van neurotransmitters door neuronen (zenuwcellen) is biologisch relevant voor de output van gedrag en de hoeveelheid bepaalt de sterkte van de respons.

Microdialyse is een veel gebruikte techniek in knaagdieren, maar wordt niet veel gebruikt in kippen. In totaal zijn er 19 studies te vinden in de literatuur, waarbij opvallend genoeg geen enkele is uitgevoerd in kippen ouder dan 25 dagen (zie **tabel 5.1**, blz 76-77). Geen van deze studies behandelt het onderwerp verenpikken.

Aangezien ernstig verenpikgedrag zich pas later in het leven ontwikkelt, vonden wij het belangrijk te weten wat de neurale afgifte is in volwassen hoog en laag verenpikkers. **Hoofdstuk 5** beschrijft het protocol dat wij ontwikkeld hebben om succesvol microdialyse uit te voeren in volwassen kippen. De *in vivo* microdialyse studie onthulde dat kippen met de neiging tot ernstig verenpikken (HFP lijn) een hogere basale of tonische 5-HT afgifte hebben dan de kippen met minder neiging tot verenpikken (LFP lijn) (zie **hoofdstuk 6**). Deze hogere afgifte in HFP kan niet verklaard worden door een grotere hoeveelheid presynaptische opslag van 5-HT, omdat na toediening van de stof d-fenfluramine (bevorderd de afgifte van 5-HT) een vergelijkbare 5-HT afgifte werd gezien in beide selectielijnen. Ook de niveaus van de 5-HT precursor tryptofaan in bloedplasma waren niet verschillend tussen lijnen. Daarom suggereren deze resultaten dat divergente genetische selectie op ernstig verenpikken het serotonerge systeem zodanig verandert dat HFP kippen een hogere tonische 5-HT afgifte hebben. Aangezien serotonine, naast dopamine, een role speelt in motor output (Jacobs et al., 2002; Jacobs and Fornal, 1997), zouden deze resultaten deels de algemeen verhoogde motorische activiteit van HFP dieren (zie **hoofdstuk 4**) kunnen verklaren. Nu microdialyse mogelijk is in volwassen kippen, valt het aan te bevelen jonge verenpikkers te vergelijken met volwassen verenpikkers om te zien of de omkering gemeten met de punch-techniek ook zichtbaar wordt in de afgifte van monoamines. Wanneer jonge genetische verenpikkers een lagere tonische afgifte laten zien, zou dit een verklaring kunnen zijn voor de gevoeligheid van het neurale systeem. Het toedienen van farmaca gericht op speciale aspecten van het neurale systeem, bijvoorbeeld selectieve monoamine heropname blokkers, specifieke receptor agonisten en antagonisten, kan eveneens meer inzicht verschaffen in de neurale achtergrond van het verenpikgedrag.

HET GENOTYPE-FENOTYPE MODEL VAN ERNSTIG VERENPIKKEN

Dit proefschrift benadrukt het belang van fenotypische discriminatie aangezien verenpikkers verschillen van slachtoffers en niet-pikkers in de activiteit van het centrale serotonerge systeem (zie **hoofdstuk 2**). Ook genetische selectie op (kenmerken gerelateerd aan) verenpikken hebben geleid tot verschillen in het serotonerge en dopaminerge systeem tussen lijnen (zie **hoofdstuk 3, 4, en 6**). Op basis van al deze bevindingen introduceer ik in **hoofdstuk 7** een model dat de ontwikkeling van het ernstig verenpikken in een groep baseert op het bestaan van een 1e en 2e order verenpikker (zie **Fig. 7.1**, blz. 119).

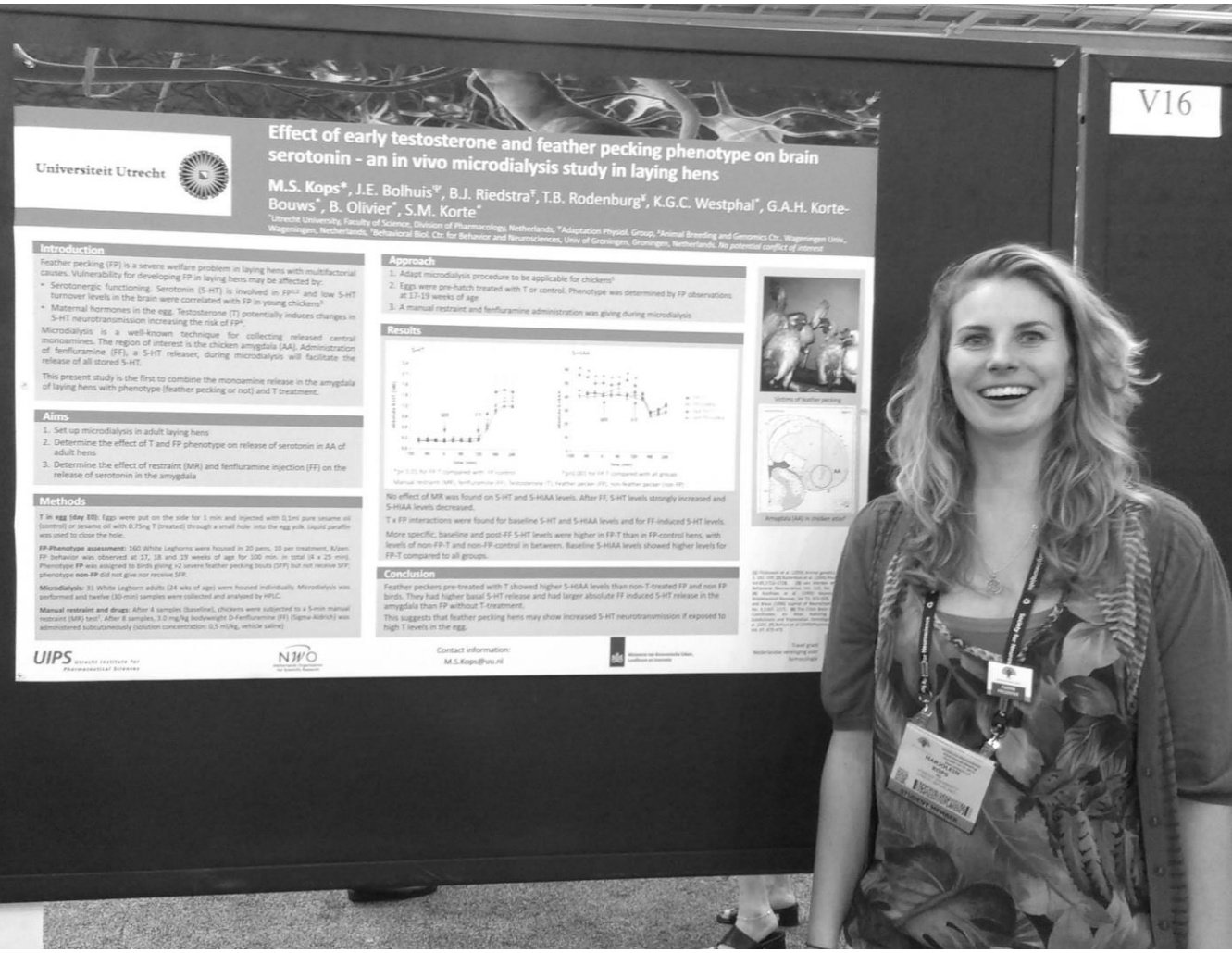
Het model wordt onderbouwd door genetische studies die stellen dat bepaalde kippen een genetische gevoeligheid hebben om zich te ontwikkelen tot verenpikker. Zo laat ik zien dat ernstig verenpikken op grotere schaal voorkomt in de HFP lijn dan in de LFP lijn (zie **tabel 4.2**, blz 60). Ook weten we dat maar een paar individuen met verenpikken beginnen (Rodenburg et al., 2013). Dit duidt erop dat niet alleen

genetische factoren maar ook omgevingsfactoren bepalen of een individu zal gaan verenpikken. Het model veronderstelt dat de impact van de genoemde omgevingsfactoren het grootst is in kippen met een genetische aanleg voor verenpikken. Deze individuen noem ik de "1e orde verenpikkers". Hierbij wordt verondersteld dat hun vermogen om te gaan met veranderende omgevingscondities bemoeilijkt wordt door hun genetische aanleg. Wanneer ernstig verenpikken is gesignaleerd in een groep, zal het aantal incidenten toenemen in de tijd omdat ook andere kippen zullen gaan pikken (zie **tabel 4.2**). De 'nieuwe' verenpikkers noem ik de "2e order verenpikkers", die voornamelijk reageren op externe stimuli zoals de beschadigde veren van slachtoffers.

Aanwezigheid van verenpikkers in een groep verandert de groepsdynamiek. Zo wordt er een toename van het angst niveau gemeten wanneer verenpikkers aanwezig zijn (de Haas et al., 2012). Ook tijd en de ontwikkeling van de dieren zelf zijn factoren van invloed op de groepsdynamiek. Deze factoren zijn gecombineerd met het model, wat leidt tot **Fig. 7.2** (zie blz 122). Deze figuur beschrijft dat onder invloed van genetische (G1) en omgevingsfactoren (E1) de 1e order verenpikker ontstaat. Dit leidt tot een veranderde omgeving (E2) waarin zich nu verenpikkers, slachtoffers en niet-pikkers bevinden. In die tweede context (E2) kunnen 2e order verenpikkers ontstaan met als gevolg een toename in slachtoffers en angstniveaus. Hoewel niet onderzocht in dit proefschrift, zal seksuele ontwikkeling en bijbehorende hormonen opnieuw tot een toename van slachtoffers kunnen leiden. Uit de praktijk is bekend dat verenpik-incidenten explosief (kunnen) toenemen rondom de periode dat kippen volgroeid raken en eieren beginnen te leggen. Het karakteriseren van verenpikkers ten behoeve van gedrags- en hersenonderzoek op dit tijdstip zal een mix aan genetische verenpikkers en fenotypische verenpikkers geven, waardoor interpretatie van de data bemoeilijkt wordt. Hoewel het karakteriseren van verenpikkers tijdrovend is, laat dit model zien dat het zeer waardevol is om de 1e order verenpikkers te onderscheiden van de 2e order verenpikkers, vanwege de verschillen in de achterliggende oorzaak van het gedrag. Daarnaast kunnen fenotype-binnen-genotype vergelijkingen verder helpen in het onderscheiden van de mogelijke neurochemische oorzaken en de gevolgen.

CONCLUSIE

De resultaten en behaalde successen in dit proefschrift laten zien dat hersenonderzoek in combinatie met gedragsonderzoek nieuwe inzichten biedt in het (vroeg) identificeren van individuen die zich zullen ontwikkelen tot verenpikker. Zowel fenotypische als genetische selectie leidt tot veranderingen in het serotonerge en dopaminerge systeem, zoals zichtbaar werd in het onderzoek van jonge en volwassen kippen. Meer onderzoek is nodig om duidelijkheid te verkrijgen over de oorzaak en het gevolg van de gemeten lage waardes in jonge verenpikkers. Microdialyse is een zeer geschikte techniek hiervoor. Het geïntroduceerde model biedt nieuwe mogelijkheden voor toekomstig onderzoek op het gebied van de samenhang tussen hersenen en gedrag. Fundamentele vragen over het neurale mechanisme achter verenpikken zullen alleen beantwoord kunnen worden wanneer onderscheid gemaakt wordt tussen 1e orde en 2e orde verenpikkers. Het model geeft tevens het belang aan van genetische selectie en omgevingsfactoren – belangrijke aspecten voor fokkers en pluimveehouders – op neurotransmitters in de hersenen en daarmee op de gevoeligheid van individuele kippen zich te ontwikkelen tot verenpikker.


V16
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Effect of early testosterone and feather pecking phenotype on brain serotonin - an in vivo microdialysis study in laying hens
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Introduction
Feather pecking (FP) is a severe welfare problem in laying hens with multifactorial causes. Vulnerability for developing FP in laying hens may be affected by:
Serotonergic functioning. Serotonin (5-HT) is involved in FP[1,2] and low 5-HT turnover levels in the brain were correlated with FP in young chickens[3]
Maternal hormones in the egg. Testosterone (T) potentially induces changes in 5-HT neurotransmission increasing the risk of FP[4].
Microdialysis is a well-known technique for collecting released central monoamines. The region of interest is the chicken amygdala (AA). Administration of fenfluramine (FF), a 5-HT releaser, during microdialysis will facilitate the release of all stored 5-HT.
This present study is the first to combine the monoamine release in the amygdala of laying hens with phenotype (feather pecking or not) and T treatment.
Aims
1. Set up microdialysis in adult laying hens
2. Determine the effect of T and FP phenotype on release of serotonin in AA of adult hens
3. Determine the effect of restraint (MR) and fenfluramine injection (FF) on the release of serotonin in the amygdala
Methods
T in egg (day 10): Eggs were put on the side for 1 min and injected with 0,1ml pure sesame oil (control) or sesame oil with 0.75ng T (treated) through a small hole into the egg yolk. Liquid paraffin was used to close the hole.
FP-Phenotype assessment: 160 White Leghorns were housed in 20 pens, 10 per treatment, R/pen. FP behavior was observed at 17, 18 and 19 weeks of age for 100 min. in total (4 x 25 min). Phenotype FP was assigned to birds giving >2 severe feather pecking bouts (SFP) but not receive SFP; phenotype non-FP did not give nor receive SFP.
Microdialysis: 31 White Leghorn adults (24 wks of age) were housed individually. Microdialysis was performed and twelve (30-min) samples were collected and analysed by HPLC.
Manual restraint and drugs: After 4 samples (baseline), chickens were subjected to a 5-min manual restraint (MR) test[5]. After 8 samples, 3.0 mg/kg bodyweight D-Fenfluramine (FF) (Sigma-Aldrich) was administered subcutaneously (solution concentration: 0.5 ml/kg, vehicle saline)
Approach
1. Adapt microdialysis procedure to be applicable for chickens[6]
2. Eggs were pre-hatch treated with T or control. Phenotype was determined by FP observations at 17-19 weeks of age
3. A manual restraint and fenfluramine administration was giving during microdialysis
Results
5-HT
5-HIAA
Time (min)
Manual restraint (MR), fenfluramine (FF), Testosterone (T), feather pecker (FP), non-feather pecker (non-FP)
No effect of MR was found on 5-HT and 5-HIAA levels. After FF, 5-HT levels strongly increased and 5-HIAA levels decreased.
T x FP interactions were found for baseline 5-HT and 5-HIAA levels and for FF-induced 5-HT levels.
More specific, baseline and post-FF 5-HT levels were higher in FP-T than in FP-control hens, with levels of non-FP-T and non-FP-control in between. Baseline 5-HIAA levels showed higher levels for FP-T compared to all groups.
Conclusion
Feather peckers pre-treated with T showed higher 5-HIAA levels than non-T-treated FP and non FP birds. They had higher basal 5-HT release and had larger absolute FF induced 5-HT release in the amygdala than FP without T-treatment.
This suggests that feather pecking hens may show increased 5-HT neurotransmission if exposed to high T levels in the egg.
Victims of feather pecking
Amygdala (AA) in chicken atlas
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NWO

List of publications

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ABSTRACTS

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Short intermezzo:

[the chickens are panicking]
Ginger: Ladies, please. Let's not lose our heads.
Bunty: Lose our heads? Aaaahh!
- Chicken Run, 2000

Dan team-microdialyse, een ‘dreamteam’: **Koen**, **Gerdien**, **Yvonne** en **Dirk**, wat hebben jullie mij geweldig geholpen! **Koen**, wie had van tevoren gedacht dat wij zo nauw moesten samenwerken? Het was maar goed dat we het met elkaar konden vinden. Zoveel details om uit te pluizen, of zal ik zeggen, vogelen, omdat wat wij wilden nog nooit was gedaan. Jouw hulp was onmisbaar, dank je wel! En onbetaalbaar was jouw blik toen je voor het eerst kippen en andere grotere diersoorten van dichtbij zag. Zoveel boerderijdieren had deze stadse jongen uit Rotterdam nog niet eerder gezien… En **Gerdien**, zonder jou geen analyse van de samples. Ook op je creativiteit maakte ik aanspraak door met hersenmateriaal van kippen aan te komen. Je slaagde met vlag en wimpel, want zelfs de kleinste hoeveelheden monoamines kon jij detecteren. Super bedankt! **Yvonne** en **Dirk**, zonder jullie had ik nooit de dieren op een diervriendelijke manier kunnen opereren. Yvonne, wat fijn dat je jouw kennis als dierenarts en ervaring met papegaaien (of, zoals jij ze noemt, patiënten) wilde delen, zodat ik leerde hoe kippen te intuberen. Dirk, vanuit Lelystad hielp je mij met de apparatuur en hielp je mee de operatiekamer in te richten. Die zag er zo “profi” uit dat iedereen daarvan onder de indruk was. **Rebecca** en **Elly**, ook jullie hebben mij

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Short intermezzo:

"Alice: How long is forever?
White Rabbit: Sometimes, just one second."
— Lewis Carroll, *Alice in Wonderland*

Terug naar Utrecht. Daar zitten nog heel veel mensen die ik wil bedanken (had ik jullie al gewaarschuwd dat het een lang dankwoord werd?).
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Rocky: You see, flying takes three things: Hard work, perseverance and... hard work.
Fowler: You said hard work twice!
Rocky: That's because it takes twice as much work as perseverance.
- Chicken Run, 2000


OODUITGANG
DEUR
RIJHOUDEN

About the author

Marjolein Kops was born on 27 March 1981 in Heerlen, the Netherlands. A year after receiving her HAVO diploma at Stella Maris College in Meerssen, she started in 1999 the higher professional education (HBO) Environmental Technology at HAS Den Bosch. Together with 3 fellow students, she was initiator of the project 'Working and Learning for Sustainable Development (WLSD): a project aimed to start a dialog between Honduran and Dutch students on sustainable development. By contacting Dutch companies and organizations, Marjolein and the fellow organizers arranged sponsoring and paid internships. These efforts resulted in a sponsored visit of 25 Dutch students to Honduran students of Zamorano, a Pan-American Agricultural School. In 2004, the project's thesis was rewarded with the "Ei van Columbus", a national price for innovative and sustainable projects. In that same year, Marjolein started the bachelor's program Biology at the University Utrecht. After graduating for her bachelor's degree in 2007 she started the master Neuroscience and Cognition in the Behavioural track. During her master's study, she conducted a research project supervised by Dr. Richard van Wezel on implied motion at Functional Neurobiology, Utrecht University. Her minor internship took place at Clinical Candidate Selection, Solvay Pharmaceuticals, Weesp under supervision of Dr. Natasja de Bruin and Dr. Lucianne Groenink (Utrecht University) on the topic of validating a preclinical rat model for cognitive deficits. Marjolein was also involved in several extracurricular activities during the master's program: she was a student's representative and student's assistant (teaching), helped organizing the symposium Mind the Brain (as secretary), and was initiator of a scientific journal of the master's program. Two volumes of this journal were published under her supervision as chief editor. She graduated in October 2009 and started her PhD-project in December 2009 at the department of Psychopharmacology under supervision of Dr. Mechiel Korte and Prof.dr. Berend Olivier in collaboration with Dr.ir Liesbeth Bolhuis of the Adaptation Physiology Group, Wageningen University and Prof.dr.dr.h.c. Onur Güntürkün of Biopsychology, Ruhr-universität Bochum. Marjolein studied the neurobiology of feather pecking in laying hens. Her work provided more insight on the biological mechanisms underlying this damaging behavior causing ongoing welfare issues in laying hens. The research performed during her PhD-project is described in this thesis.

“Begin at the beginning," the King said, very gravely, "and go on till you come to the end: then stop.”
— Lewis Carroll, *Alice in Wonderland*

But I say, don’t stop. Keep on exploring, investigating and developing!
That’s my way of living, *Marjolein*