IMPULSIVE-AGGRESSIVE PHENOTYPES IN MALE DARK AGOUTI RATS:

NEURONAL CORRELATES AND EFFECTS ON MONOAMINERGIC RECEPTORS

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Abstract

Impulsivity and aggression are essential components of the natural repertoire of personality traits in humans and animals. In everyday life, individuals are faced with environmental challenges which require appropriate and well-adapted behavioral responses. The display of impulsive and aggressive behaviors within an adaptive range can help the individual to cope with these challenges. In humans, impulsive and aggressive behaviors are of particular interest when they occur in pathological form or when they pose a problem to society. Impairments in the adaptive ranges of these behaviors can contribute to personality problems or neuropsychiatric disorders including addictions, substance abuse, pathological gambling or attention deficit/hyperactivity disorder (ADHD). Even though impulsivity and aggression have long been associated with each other in many species, there are still many open questions concerning their relationship, especially in view of the multifaceted nature of these behaviors.

In **chapter 2**, the behavioral profiles of choice impulsivity and territorial aggression were characterized in healthy individuals of the Dark Agouti (DA) inbred rat strain and it was investigated whether or not an association between these behaviors exists in these rats. Impulsivity is generally described as the tendency to act without forethought and it typically comprises impulsive choice behavior, characterized as intolerance towards delayed rewards, and impulsive action, described as the inability to inhibit motor responses. In DA rats, impulsive choice was assessed by use of a delay-discounting (DD) task which requires the animal to select between a small, immediate reward and a larger, but delayed reward. Thereby, impulsive individuals tend to prefer the immediate reward option and devalue a large reward with increasing waiting time. Aggressive behavior in DA rats was assessed by use of a resident-intruder (RI) task, in which a resident rat with an established territory is confronted with an unfamiliar opponent. During such inter-male confrontations, species-typical offensive behaviors are displayed with the purpose to protect mating partners and to retain food sources. In this regard, aggressive behavior represents an important form of social communication. Overall, a wide range of individual differences for choice impulsivity was found in male DA rats, whereas aggressive behavior was confined to low and medium levels. Although several combinations of impulsive and aggressive phenotypes were detected in the DA strain, an overall correlation for impulsivity and aggression was not observed. Therefore, these findings are opposed to the widespread theory that increased levels of impulsivity are linked to increased aggression.

The neuronal correlates that underlie impulsive choice behavior and territorial aggression were the main focus of **chapter 3** of the present thesis. It was investigated to which extent DA rats with varying levels of impulsivity and aggression differed in terms of neuronal activation patterns in selected brain regions. This was achieved by immunohistochemical detection of the c-Fos protein, a common marker for the identification of activated neurons in response to behavioral performance. An additional aim was to examine potential changes in the number of neurons expressing the serotonin 5-HT_{1A} receptor induced by DD or RI task performance. The serotonergic system in general, and the 5-HT_{1A} receptor in particular, play an important role in the modulation of impulsivity and aggression.
It was found that the frontal cortex, which is involved in a variety of executive and cognitive functions, was inhibited in rats that show increased choice impulsivity as well as in rats with low levels of aggression. This indicates an involvement of the frontal cortex in the regulation of both behaviors. Beyond this finding, there was only little evidence for a shared neuroanatomical basis for choice impulsivity and territorial aggression. While effects specifically associated with low aggressive behavior were additionally observed in the amygdala, a limbic structure implicated in the evaluation of social

interactions, this brain region was not affected by the DD task. For the 5-HT_{1A} receptor, alterations were observed within the dorsal raphe nucleus (DRN), a major output source for serotonin, but these effects were not specific for impulsivity or aggression level, indicating an involvement in general behavioral processes linked to the execution of both tasks.

Chapter 4 presents further investigations on distinct levels of impulsive choice behavior in DA rats at the neurochemical level. Expression levels of the 5-HT_{1A} receptor and the dopamine D2 receptor were analyzed and compared for low- and highly-impulsive individuals by quantification of two parameters. These were the number of neurons expressing each receptor and the density of each receptor assessed independently of neuron number. The 5-HT_{1A} and D2 receptors are among the most abundant and widely distributed monoaminergic receptor subtypes in the brain. Depending on the locus of action, both receptors can exert inhibitory effects on neuronal signaling within the target areas of serotoninergic and dopaminergic projections, or they can directly affect neurotransmitter release at the site of synthesis. Therefore, changes in receptor expression were examined in cortical areas, the nucleus accumbens (NAc) and the amygdala, which all receive serotonergic and dopaminergic input, as well as in the ventral tegmental area and the substantia nigra pars compacta, two output structures for dopamine, and the DRN as a source for serotonin. The main finding for 5-HT_{1A} was a reduction in receptor expression in low-impulsive rats within the anterior cingulate cortex. This indicates that an enhanced ability to tolerate delayed rewards is associated with a reduced activity of this serotonergic receptor in a specific subregion of the frontal cortex. Alterations in D2 receptor expression were primarily observed for highly-impulsive individuals, such that expression levels were decreased in the prelimbic cortex, another subregion of the frontal cortex, and in the NAc. This indicates that a decreased D2 receptor function contributes to an intolerance towards delayed rewards. As a whole, these findings demonstrate a differential involvement of 5-HT_{1A} and D2 receptors in neurochemical processes underlying self-controlled responding and delay aversion.

Zusammenfassung

Impulsivität und Aggression sind wesentliche Bestandteile des natürlichen Spektrums von Persönlichkeitsmerkmalen bei Mensch und Tier. Im täglichen Leben, sieht sich jedes Individuum durch seine Umwelt vor Herausforderungen gestellt, die adäquate und gut angepasste Reaktionen und Verhaltensweisen erfordern. Das Zeigen von impulsivem und aggressivem Verhalten innerhalb eines adaptiven Bereichs kann dem Individuum dabei helfen, diese Herausforderungen zu bewältigen. Beim Menschen sind impulsive und aggressive Verhaltensweisen besonders von Interesse, wenn diese in pathologischer Form auftreten oder wenn sie ein Problem für die Gesellschaft darstellen. Beeinträchtigungen in der adaptiven Spanne dieser Verhaltensweisen können zu Persönlichkeitsproblemen oder neuropsychiatrischen Störungen beitragen, wie Suchtverhalten, Drogenmissbrauch, pathologischem Glücksspiel oder der Aufmerksamkeitsdefizit-/Hyperaktivitäts-störung (ADHS). Auch wenn Impulsivität und Aggression bei vielen Spezies seit langem miteinander assoziiert werden, sind noch viele Fragen bezüglich der Zusammenhänge beider Verhaltensweisen ungeklärt, insbesondere angesichts ihrer Vielschichtigkeit.

In Kapitel 2 wurden die Verhaltensprofile des impulsiven Wahlverhaltens und der territorialen Aggression bei gesunden Individuen des Dark Agouti (DA) Rattenstamms charakterisiert, und es wurde untersucht, ob ein Zusammenhang zwischen diesen Verhaltensweisen besteht. Impulsivität wird im Allgemeinen als die Tendenz beschrieben, ohne Voraussicht zu handeln, und es umfasst typischerweise impulsives Wahlverhalten, das als Intoleranz gegenüber verzögerten Belohnungen gekennzeichnet ist, und motorische Impulsivität, die als Unfähigkeit beschrieben wird, motorische Handlungen zu unterdrücken. Bei DA-Ratten wurde das impulsive Wahlverhalten mit Hilfe eines Delay-Discounting-Paradigmas (DD) untersucht, bei dem die Tiere zwischen einer kleinen, sofortigen Belohnung und einer größeren, aber verzögerten Belohnung wählen können. Dabei neigen impulsive Individuen dazu, die sofortige Belohnungsoption zu bevorzugen und eine große Belohnung mit zunehmender Wartezeit abzuwerten. Aggressives Verhalten bei den DA-Ratten wurde mit Hilfe des sogenannten „Resident-Intruder“-Tests untersucht, bei dem der Bewohner eines etablierten Territoriums mit einem unbekannten Eindringling konfrontiert wird. Bei Konfrontationen zwischen männlichen Ratten werden spezies-typische offensive Verhaltensweisen gezeigt, mit dem Zweck, Sexualpartner und Nahrungsquellen zu verteidigen. In dieser Hinsicht stellt das aggressive Verhalten eine wichtige Form der sozialen Kommunikation dar. Die Ergebnisse der Verhaltensuntersuchungen bei männlichen DA-Ratten zeigten ein breites Spektrum individueller Unterschiede im impulsiven Wahlverhalten der Tiere, während aggressives Verhalten auf niedrige und mittlere Werte begrenzt war. Obwohl beim DA-Stamm mehrere Kombinationen von impulsiven und aggressiven Phänotypen festgestellt werden konnten, wurde keine signifikante Korrelation zwischen Impulsivität und Aggression beobachtet. Daher stehen diese Ergebnisse im Widerspruch zu der weit verbreiteten Theorie, dass ein erhöhtes Maß an Impulsivität mit erhöhter Aggression einhergeht.

Die neuronalen Strukturen, die dem impulsiven Wahlverhalten und der territorialen Aggression zugrunde liegen, standen im Fokus von **Kapitel 3** der vorliegenden Arbeit. Es wurde untersucht, inwieweit sich DA-Ratten mit unterschiedlichen Ausprägungen von impulsivem und aggressivem Verhalten hinsichtlich der neuronalen Aktivierungsmuster in ausgewählten Hirnregionen unterscheiden. Hierfür wurde der immunhistochemische Nachweis des c-Fos-Proteins genutzt, einem gängigen Marker für die Identifizierung aktivierter Neurone als Reaktion auf ausgeübtes Verhalten.
Ein weiteres Ziel war die Untersuchung möglicher Veränderungen in der Anzahl der Neurone, die den Serotonin-5-HT_{1A}-Rezeptor exprimieren, die durch die Durchführung von DD- oder RI-Tests induziert

werden. Das serotonerge System im Allgemeinen und der 5-HT_{1A}-Rezeptor im Besonderen spielen eine wichtige Rolle bei der Modulation von Impulsivität und Aggression.
Es wurde festgestellt, dass der frontale Kortex, der an einer Vielzahl exekutiver und kognitiver Funktionen beteiligt ist, bei Ratten inhibiert war, die ein erhöhtes impulsives Wahlverhalten aufweisen, sowie bei Ratten mit einem geringen Grad an Aggression. Dies deutet auf eine Beteiligung des frontalen Kortex an der Regulierung beider Verhaltensweisen hin. Abgesehen von diesem Ergebnis gab es nur wenige Hinweise auf eine gemeinsame neuroanatomische Grundlage für impulsives Wahlverhalten und territoriale Aggression. Während zusätzliche Effekte, die speziell mit geringem aggressivem Verhalten assoziiert sind, in der Amygdala beobachtet wurden, einer limbischen Hirnregion, die an der Bewertung sozialer Interaktionen beteiligt ist, war dieses Areal durch die Ausübung des DD-Tests nicht beeinflusst. Beim 5-HT_{1A}-Rezeptor wurden Veränderungen im dorsalen Raphe-Kern (DRN) beobachtet, einem der wichtigsten Syntheseorte von Serotonin im Gehirn, jedoch waren diese Effekte nicht spezifisch für das Impulsivitäts- oder Aggressionslevel, was auf eine Beteiligung an allgemeinen Verhaltensprozessen im Zusammenhang mit der Ausführung beider Verhaltenstests hindeutet.

In **Kapitel 4** werden weitere Untersuchungen zu den verschiedenen Ausprägungen des impulsiven Wahlverhaltens bei DA-Ratten auf neurochemischer Ebene dargestellt. Die Expressionsraten des 5-HT_{1A}-Rezeptors und des Dopamin-D2-Rezeptors wurden bei wenig und hoch impulsiven Individuen durch die Quantifizierung von zwei Parametern analysiert und miteinander verglichen. Dabei wurde die Anzahl der Neurone, die den jeweiligen Rezeptor exprimieren, und die Dichte jedes Rezeptors unabhängig von der Anzahl der Neurone bestimmt. Die 5-HT_{1A}- und D2-Rezeptoren gehören zu den monoaminergen Rezeptorsubtypen mit dem häufigsten Vorkommen und der weitesten Verbreitung im Gehirn. Je nach Wirkungsort können beide Rezeptoren eine hemmende Wirkung auf die neuronale Signalübertragung in serotonergen und dopaminergen Projektionengebieten ausüben, oder sie können die Neurotransmitterfreisetzung am Ort der Synthese direkt beeinflussen. Daher wurden Veränderungen der Rezeptorexpression sowohl in kortikalen Arealen, dem Nucleus accumbens (NAc) und der Amygdala, die alle serotonergen und dopaminergen Input erhalten, untersucht, sowie im ventralen tegmentalen Areal und der Substantia nigra pars compacta, zwei Syntheseorten von Dopamin, und dem DRN als Quelle für Serotonin. Das Hauptergebnis für den 5-HT_{1A}-Rezeptor war eine Verringerung der Expression bei wenig impulsiven Ratten im anterioren cingulären Cortex. Dies deutet darauf hin, dass eine gesteigerte Fähigkeit, verzögerte Belohnungen zu tolerieren, mit einer verringerten Aktivität dieses serotonergen Rezeptors in einer spezifischen Subregion des frontalen Cortex verbunden ist. Veränderungen in der D2-Rezeptorexpression wurden vor allem bei hoch impulsiven Ratten beobachtet, wobei die Expressionswerte im prälimbischen Cortex, einer weiteren Subregion des frontalen Cortex, und im NAc verringert waren. Dies weist darauf hin, dass eine verminderte D2-Rezeptorfunktion zu einer Intoleranz gegenüber verzögerten Belohnungen beiträgt. Insgesamt belegen diese Ergebnisse eine unterschiedliche Beteiligung von 5-HT_{1A}- und D2-Rezeptoren an neurochemischen Prozessen, die der Selbstkontrolle und der Verzögerungsaversion zugrunde liegen.

Chapter 1

General introduction

1.1. Definition of impulsivity

Impulsivity is an important element in everyday life for both humans and non-human animals. Its behavioral manifestations are multidimensional and depend on the conditions of the situation to react on. A general definition was given by Evenden in 1999 by the following words: "*A wide range of actions that are poorly conceived, prematurely expressed, unduly risky, or inappropriate to the situations and that often result in undesirable outcomes*". This delineation already implies the heterogenous nature of impulsive behavior and additionally indicates its distinct behavioral aspects. These aspects encompass unplanned or risky behavior with regard to outcome expectancy, the tendency to act without forethought as well as the inability to inhibit motor responses (Evenden, 1999; Dalley and Roiser, 2012; Smith et al., 2015). By now, impulsivity is commonly divided into two main classifications, namely impulsive choice, also entitled impulsive decision-making, and impulsive action or motor impulsivity.

Studies in rodents have found that impulsive choice and action are most often uncorrelated as assessed by a variety of behavioral paradigms (Winstanley et al., 2004a; van den Bergh et al., 2006b; Broos et al., 2012; Simon et al., 2013) and that the neuroanatomical and -chemical basis for both aspects shows only partial overlap (Dalley et al., 2011; Nautiyal et al., 2017).

Choice impulsivity is particularly characterized by disrupted evaluation of delayed outcomes and it is expressed when two options of reinforcement differ in their overall value and the time point of acquisition. Impulsive individuals thereby devalue a more favorable reward the longer the waiting time and tend to prefer the immediate option (Odum, 2011; Robbins and Dalley, 2017).

By contrast, impulsive action is described as mistimed response behavior with the difficulty to suppress the urge of a reaction prior to or during certain situations. It can manifest as premature or anticipatory responses as well as the failure to cancel already initiated actions (Dalley and Roiser, 2012).

Thus, impulsive behavior can result in disadvantages that prevent the individual to receive a relatively more valuable reinforcement. In dangerous situations, however, it can be necessary and more beneficial to react rapidly in order to avoid personal harm.

Accordingly, impulsive behavior is expressed in an adaptive range, and it represents a characteristic in the natural scope of personality traits (Evenden, 1999; Dalley et al., 2011).

Impairments in this adaptive range of impulse control can result in or contribute to personality disorders which in humans often include addictions, gambling disorders or attention deficit/hyperactivity disorder (ADHD) (Winstanley, 2011; Robbins and Dalley, 2017).

Impulsivity in general underlies multiple functional mechanisms with regard to timing and reward evaluation (Dalley et al., 2004; Marshall et al., 2014; Smith et al., 2015; Marshall and Kirkpatrick, 2016). The present thesis focusses on impulsive choice behavior in rodents and therefore its functional mechanisms will be described in more detail with respect to delayed reinforcement tasks.

1.2. Testing choice impulsivity

In rodents, impulsive choice is classically assessed using inter-temporal choice tasks including delay-discounting (DD) procedures which require the animal to repeatedly decide between a small, immediate reward and a larger, but delayed reward (Evenden and Ryan, 1996). These paradigms are implemented using operant conditioning to establish associations between a response, for example a lever press, and the obtaining of a reward, such as food. The term discounting refers to the devaluation of a large reward with increasing waiting time (delay) and corresponds to the increased preference for the immediate reward (Odum, 2011; Robbins and Dalley, 2017).

The design of DD paradigms is mainly adapted with regard to two parameters, delay length and reinforcer size, and thus relates to temporal processes and processing of reward (e.g., magnitude discrimination) (Dalley et al., 2004; Marshall et al., 2014; Smith et al., 2015; Marshall and Kirkpatrick, 2016).

Temporal processes include the ability to correctly estimate the length of durations. For DD tasks this means to perceive the length of a delay correctly in order to decide whether or not it is subjectively tolerable to wait for a large reward. Poor timing accuracy in this context may lead to the overestimation of delays and may enhance intolerance towards delayed rewards in impulsive individuals (Galtress et al., 2012; Marshall et al., 2014; Smith et al., 2015; Marshall and Kirkpatrick, 2016).

Moreover, timing abilities are crucial for the apprehension of events that occur or need to be executed in sequence (Kolb, 1984; Dalley et al., 2004). To estimate choice impulsivity, DD tasks are performed in several sessions with a stepwise increase in delay length within each session and with discrete trials for each choice. For the subject, it is necessary to recognize the temporal order of trials such that each trial represents a new choice between both reward options, and additionally that the large reward is increased as the session progresses.

The second major component of mechanisms that underlie choice impulsivity refer to the processing of reward (Acheson et al., 2006; Marshall and Kirkpatrick, 2016). The evaluation of reward magnitudes is thereby essential for the discrimination of reward valences in discriminative tasks. Previous research has shown that reward preference is enhanced when reward size increases as well, and that the tolerance towards large delays may be linked to better magnitude discrimination abilities (Marshall and Kirkpatrick, 2016). In a DD task, the subjective value of a large reward may therefore be perceived as greater in individuals that display low levels of impulsivity.

In addition to reinforcer magnitude sensitivity, reward processing also involves reward learning, reward expectation and reward waiting, which are processes that are relevant at varying time points of DD tasks (Cardinal et al., 2001; Izquierdo, 2017; Stalnaker et al., 2018).

Other commonly used variations of inter-temporal choice tasks are adjusting procedures during which the animals' responses directly affect the adjusted parameter, may it be delay length or reward size (Mazur, 1987; Moschak and Mitchell, 2014). While adjusting procedures can be used for the separate evaluation of delay or reward magnitude sensitivities (Saddoris et al., 2015), DD tasks imply differences in both of these parameters and thus allow the assessment of choice impulsivity per se (Winstanley et al., 2006b).

In the current thesis a DD paradigm will be used to investigate individual differences in impulsive choice behavior in male inbred rats.

1.3. Aggressive behavior

In many species, a behavioral trait often associated with impulsivity is aggressive behavior. In humans, together with increased impulsivity, aggression is part of the symptomatology of psychiatric disorders such as substance abuse or ADHD. Moreover, aggression explicitly contributes to impulse control disorders in which impulsivity is a key symptom (Siever, 2008). Despite these contributions of maladaptive forms of aggression in psychopathology, aggression is a common component of the natural behavioral repertoire of healthy individuals and it is therefore widely studied in humans and other animals in order to elucidate the underlying processes of this trait (Natarajan and Caramaschi, 2010; Takahashi and Miczek, 2014).

Aggression is generally defined as agonistic behavior that causes personal harm to oneself or others, but which may serve different purposes (Nelson and Trainor, 2007; Wrangham, 2018). In humans, forms of aggression are usually classified into two major categories: instrumental and impulsive. Instrumental aggression is described as proactive, premeditated or controlled behavior with a specific goal in mind (Neumann et al., 2010; Rosell and Siever, 2015; Wrangham, 2018). In the animal kingdom, this purposeful type of aggressive behavior is difficult to assess objectively, but, as an example, it can be observed with intentional attacks of a predator on prey (Tulogdi et al., 2010).

Impulsive aggression, on the contrary, is characterized as reactive, hostile and affective behavior which may be induced by provocation or stress or may be driven by negative emotional states such as anger or fear (Neumann et al., 2010; Siever, 2008). An equivalent to this type of aggression, as defined for humans, can also be found in animals in form of offensive aggression (Cervantes and Delville, 2007). It is often observed in situations in which individuals compete for food, defend their territory (territorial aggression) or protect their offspring from potential dangers (maternal aggression). It is additionally important for the establishment or maintenance of hierarchical structures. Thus, aggressive behavior represents an essential aspect of social communication and an effective element to ensure survival and reproduction (Takahashi and Miczek, 2014; Tulogdi et al., 2015; Wrangham, 2018).

1.4. Testing territorial aggression

In the present thesis, the investigation of inter-male territorial aggression is of particular interest, thus, a standardized model in rodents, the resident-intruder (RI) paradigm, is applied to test for this type of social interaction (Koolhaas et al., 2013).

In this approach, the resident animal is provided with resources which are necessary for the establishment of a territory, i.e., it is given access to a mating partner, food and the territory itself (home cage). For test conditions, the female, food and housing are removed from the home territory and the resident is confronted with an unfamiliar intruder. This confrontation typically elicits a variety of species-specific social behaviors. The resident will defend its territory and resources against the opponent by the display of offensive behaviors including threatening gestures, chase or physical attacks (Koolhaas et al., 2013; Takahashi and Miczek, 2014). Threat behavior usually precedes physical attacks and expresses aggressive intentions which enable the opponent to withdraw and to reduce the risk for physical harm (Haller, 2018).

The expression of offensive behaviors is regulated by inhibitory control mechanisms in order to maintain it in an adaptive range. Therefore, differences in the individual level of aggression are found in several rodent species (Blanchard et al., 1988; de Boer et al., 2003; Cervantes and Delville, 2007).

In the present thesis, the RI paradigm is used to investigate the individual range of territorial aggression and its relationship to impulsive choice behavior in rats.

1.5. Impulsive-aggressive phenotypes in rodents

Associations of impulsive and aggressive behaviors expressed in rodents were previously reported for both aspects of impulsivity, impulsive choice and impulsive action.

In rats and hamsters, the level of territorial aggression was found to increase along with impulsive choices in delayed-reward tasks (van den Bergh et al., 2006b; Cervantes and Delville, 2007; Rudebeck et al., 2007). These studies therefore indicate the involvement of delayed decision-making in offensive aggression during inter-male confrontations. In this context, aggression in response to the invasion of a territory might be a consequence of poor behavioral choices, potentially due to a failure to assess the opponent's behavior and to initiate an appropriate response (van den Bergh et al., 2006b; Blair, 2016). For instance, rapid, unplanned attacks (short attack latency) or a high number of physical attacks might be suboptimal when an opponent engages in submissive postures quickly after the resident has signaled its aggressive intentions by threatening behavior. This is relevant because the expression of offensive acts and especially physical attacks is associated with high energy consumption and an increased risk of injuries (Haller, 2017).

Nevertheless, an inverse correlation between choice impulsivity and aggression, i.e., increased aggression and less impulsive choice, was found in a rat line selected for novelty-induced locomotion (Flagel et al., 2010; Kerman et al., 2011).

Regarding impulsive action, studies using transgenic models or rodent selection lines have found positive correlations with aggression (Flagel et al., 2010; Kerman et al., 2011; Nautiyal et al., 2015). These findings suggest the inability to inhibit aggressive responses associated with social confrontations and accordingly a possible involvement of motor impulsivity (van den Bergh et al., 2006b; Bevilacqua et al., 2010). In contrast to this assumption, a lack of correlation with impulsive action has been observed for Wistar rats (van den Bergh et al., 2006b) and for wild-type rats with a normal range of aggression, whereas excessive aggression in this wild-type strain was positively correlated to motor impulsivity (Coppens et al., 2014).

Thus, besides the type of impulsivity, several factors appear to have an impact on impulsive-aggressive interactions, such as genetic factors or maladaptive levels of aggression (for review see: Bevilacqua and Goldman, 2013; Miczek et al., 2015; Veroude et al., 2016).

In summary, both types of impulsivity were previously associated with territorial aggression, although contradictory findings were observed with regard to the direction of the correlation. Moreover, impulsive choice and action were found to be distinct behavioral entities (Winstanley et al., 2004a; van den Bergh et al., 2006b; Broos et al., 2012; Simon et al., 2013) with only partial overlap at the neuroanatomical and -chemical level (Dalley et al., 2011; Nautiyal et al., 2017). Hence, each impulsivity aspect appears to differentially relate to the aggressive trait.

The main aim of this thesis is to study the individual expression of choice impulsivity and territorial aggression in rats and to test the hypothesis that both traits are positively correlated in an inbred strain.

Furthermore, many earlier studies have identified several brain structures and neurotransmitters, such as serotonin and dopamine, involved in the regulation and modulation of impulsivity and aggression (Winstanley et al., 2005). In the present thesis, neuronal activation patterns and the distribution of relevant monoaminergic receptors will be investigated following behavioral examination. Therefore, neuroanatomical correlates of both behaviors and the involvement of monoaminergic neurotransmitter systems will be presented in the following paragraphs.

1.6. Neuronal correlates of impulsive choice behavior

Among the key structures that are implicated in choice impulsivity are the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC), two distinct areas of the frontal cortex located in the rostral portion of the frontal lobe. Subcortical structures particularly include amygdala, hippocampus, and the nucleus accumbens (NAc), with the latter divided into a core (AcbC) and shell (AcbSh) region. The subthalamic nucleus (STN) of the diencephalon, the mesencephalic ventral tegmental area (VTA) and the raphe nuclei of the brainstem are further parts of the functional network (Fig. 1.1; for review see: Baunez and Lardeux, 2011; Cardinal, 2006; Dalley et al., 2011). For an overview of brain regions involved in choice impulsivity see figure 1.1. The contribution of some of these brain areas to impulsive choice behavior is still under debate due to contradictory findings, especially regarding the role of mPFC and OFC, as well as the AcbSh or the substantia nigra pars compacta (SNc), a mesencephalic brain structure located adjacent to the VTA.
In the current thesis, the involvement of several of these brain regions or selected subregions in choice impulsivity will be investigated immunohistochemically by expression levels of the c-Fos protein, which is used as a marker for neuronal activity in response to behavioral performance (Okuno, 2011).
The following sections describe in more detail the functions and connectivity of the above-mentioned brain regions and illustrate their specific involvement in impulsive choice behavior.

1.6.1. The frontal cortex

The frontal cortex is generally involved in a variety of executive and higher-order cognitive functions including goal-directed behavior, attention, learning and memory (working memory), cognitive flexibility and inhibitory control (Winstanley et al., 2006a; Schoenbaum et al., 2011; Fitoussi et al., 2015).
The OFC constitutes the ventral portion of the frontal cortex and, along a lateral-to-medial axis, it can be anatomically divided into dorsolateral, lateral, ventral and medial portions (DLO, LO, VO, MO, respectively). The mPFC lies adjacent to the MO along the medial wall and constitutes three anatomical subdivisions: infralimbic (IL), prelimbic (PrL) and anterior cingulate (Cg) cortices (Paxinos and Watson, 1997; Vertes, 2004).
Implications of the OFC in impulsive choice behavior were shown by enhanced neuronal activation following completion of a delay-discounting task (da Costa Araújo et al., 2010). Lesion studies support the involvement of the OFC, although its inactivation resulted in both increased (Mobini et al., 2002; Rudebeck et al., 2006) and decreased levels of choice impulsivity (Winstanley et al., 2004b; Mar et al., 2011). There are also studies which report no effect on choice behavior following OFC lesion (Mariano et al., 2009; Abela and Chudasama, 2013) or selective inactivation of the LO subregion, which additionally had no influence on separate assessment of delay and magnitude sensitivities (Moschak and Mitchell, 2014). Previous work supporting the contribution of the OFC in choice impulsivity have suggested a specific role in the integration of reinforcer value (Mobini et al., 2002; Cardinal, 2006; Winstanley et al., 2006b; Fitoussi et al., 2015), value representation during task performance (Schoenbaum et al., 2011; Rudebeck and Murray, 2014) and evaluation of reward outcome (Wallis, 2012).
In comparison, the mPFC was predominantly associated with temporal reward processing such as assessment of the order of events (Dietrich and Allen, 1998; Cardinal et al., 2001; Winstanley et al., 2006b; Cassaday et al., 2014) or reward-related learning (Cardinal, 2006).

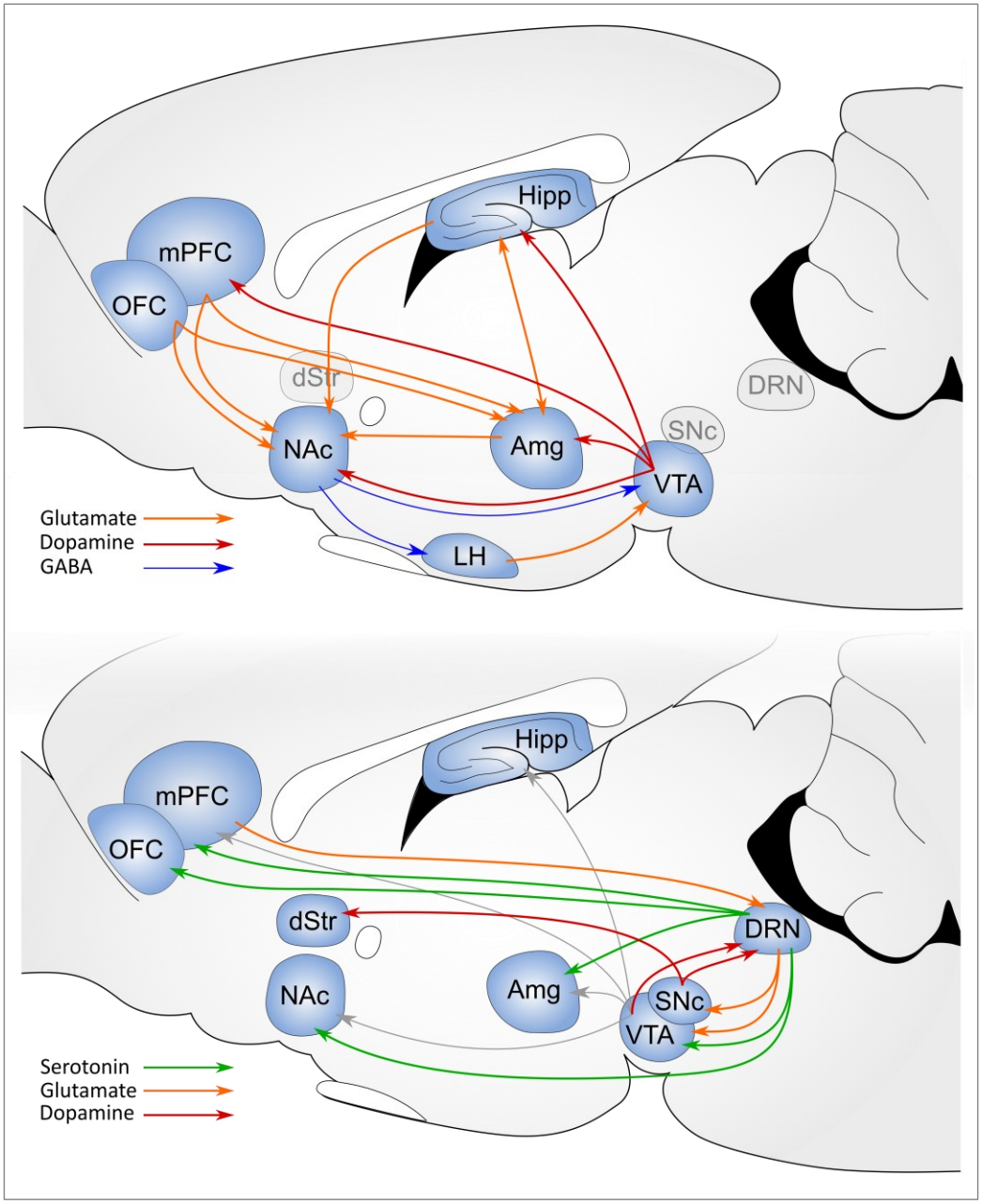

Figure 1.1 | **Neuroanatomical pathways and structures involved in impulse control in the rodent brain.** A simplified schematic of the limbic corticostriatal loop regulating reward-related behavior is shown in the **upper panel**. Top-down cognitive control is mediated by frontal cortical areas (OFC, mPFC) via glutamatergic connections to limbic, subcortical structures, such as nucleus accumbens (NAc) and amygdala (Amg). The NAc is highly interconnected with other areas of the limbic corticostriatal loop and represents a key structure in the regulation of reward and motivational processes. GABAergic projections from the NAc reach the ventral tegmental area (VTA) via a direct pathway and indirectly via the ventral pallidum (not shown). NAc and VTA are also indirectly connected via the lateral hypothalamus (LH). The VTA is a major output source for dopamine and projects back to mPFC (mesocortical pathway), as well as hippocampus (Hipp), Amg and NAc (mesolimbic pathway). Areas shaded in gray indicate additional brain regions linked to impulsivity (see lower panel). The **lower panel** represents a simplified overview of afferent and efferent connections of the dorsal raphe nucleus (DRN), a major output source for the neurotransmitter serotonin. Important serotonergic projections reach frontal cortex, NAc and Amg. Arrows shaded in gray indicate dopaminergic projections already presented in the upper panel. dStr, dorsal striatum; OFC, orbitofrontal cortex; mPFC, medial prefrontal cortex; SNc, substantia nigra pars compacta. The presented graphic is based on findings reviewed by Cardinal (2006), Dalley et al. (2011), Hu (2016), Russo and Nestler (2013) and Zhang (2020).

In contrast to the OFC, lesions of the mPFC and selective lesions of the Cg had no effect on choice impulsivity (Cardinal et al., 2001; Rudebeck et al., 2006) and neuronal activation levels were unaffected by delayed reinforcement (da Costa Araújo et al., 2010). However, more differentiated findings were observed by Winstanley and colleagues using in vivo microdialysis. The authors found that delay-discounting performance induced dopamine release in the OFC and a combined release of dopamine and serotonin in the mPFC, implicating both areas in choice impulsivity with potentially distinct functional contributions. As a result, the mPFC was suggested to mediate reward acquisition, whereas both regions seemed to contribute to choice behavior in general (Winstanley et al., 2006b).

1.6.2. The limbic corticostriatal loop

With regard to connectivity**,** the mPFC was often reported to be involved in reward circuits which additionally include the NAc, VTA, hippocampus, amygdala and hypothalamus (Fig. 1.1; for review see: de Boer et al., 2017; Hu, 2016; Russo and Nestler, 2013; Zhang, 2020). This reward circuitry is also known as the limbic corticostriatal loop due to its involved brain regions (Winstanley, 2007; Roesch and Bryden, 2011).
Cortical connections arise in the mPFC which sends glutamatergic projections to limbic structures such as ventral striatum (NAc) and the amygdala. The NAc in turn connects to the VTA via two pathways, directly through GABAergic innervations and indirectly via the ventral pallidum (VP) and the lateral hypothalamus (LH). The VP then sends GABAergic projections to the VTA, while inputs from the LH are mainly glutamatergic. The VTA is located in the ventral mesencephalon and represents one of the major dopaminergic output sources in the brain. These dopaminergic VTA neurons project back to the mPFC, forming the mesocortical pathway, while additional dopaminergic projections reach NAc, amygdala and hippocampus, which constitute structures of the mesolimbic system. The amygdala and the hippocampus further close the limbic corticostriatal loop by projecting back to the NAc via glutamate (for review see: Hu, 2016; Russo and Nestler, 2013; Zhang, 2020).
Similar to the mPFC, the OFC is frequently considered as part of the limbic corticostriatal loop and associated with the regulation of reward-related behavior (Schoenbaum et al., 2006; Winstanley, 2007; Stalnaker et al., 2018). In this regard, the OFC sends glutamatergic efferents to the AcbC and AcbSh (Winstanley et al., 2006b), the basolateral amygdala (BLA) (Schoenbaum et al., 2006) and to dopaminergic neurons in the VTA (Hu, 2016).
Although OFC and mPFC overall connect to similar brain regions, tracing studies have shown that subregions of OFC and mPFC differ in projection sites as well as density of projections to different brain regions involved in inter-temporal choice (Hoover and Vertes, 2007; Babalian et al., 2019).
Thus, contrasting behavioral effects may be explained by variations in the localization and the size of frontal cortex lesions (Churchwell et al., 2009; Mar et al., 2011). Moreover, changes in the functional connectivity of components of the reward pathway may be reflected in individual differences in trait-like choice impulsivity (Barlow et al., 2018).

1.6.3. Subcortical structures of the reward circuitry

One major subcortical structure implicated in impulsive behavior in rodents is the amygdala, a heterogeneous brain region located in the temporal lobe and composed of several subnuclei. The amygdala is known to process a variety of sensory stimuli and emotional states to evaluate social

behavior (Linley et al., 2017), and it plays an important role in emotional learning and memory (O'Connell and Hofmann, 2011; Maeng and Shors, 2013; Nakamura, 2013).
Especially the BLA has been associated with choice impulsivity as lesioning of this area increased the preference for immediate versus delayed rewards (Winstanley et al., 2004b; Churchwell et al., 2009). Specific reward-related functions of this amygdalar subregion were found to be reward-learning (Winstanley et al., 2004b, 2006a) and assessment of reward value (Fitoussi et al., 2015), similar to functions reported for the OFC, from which the BLA receives afferent projections (Schoenbaum et al., 2006; Zhang, 2020).
The BLA also shares connections with the hippocampus and the NAc, two subcortical limbic structures implicated in reward-related functions (Zhang, 2020). Lesions of both of these brain regions have been found to enhance impulsive choice in delayed-reward tasks (Cardinal et al., 2001; Cheung and Cardinal, 2005; Pothuizen et al., 2005; Bezzina et al., 2007; McHugh et al., 2008; da Costa Araújo et al., 2009; Mariano et al., 2009; Galtress and Kirkpatrick, 2010; Valencia-Torres et al., 2012; Abela and Chudasama, 2013; Feja et al., 2014), although accumbal core and shell regions have shown conflicting results (Pothuizen et al., 2005; Acheson et al., 2006; Feja et al., 2014; Moschak and Mitchell, 2014).
In more detail, the hippocampus is a temporal lobe structure, which is important for the processing and retention of spatial and temporal information to support orientation, memory formation and aspects of associative learning, including reward-related learning (Wikenheiser and Schoenbaum, 2016; Ikekubo et al., 2020). A recent electrophysiologic study, for instance, identified that hippocampal neurons process information on both delay and reward, such as encoding of delay length or the presentation of reward value in the context of reward expectation (Masuda et al., 2020). Hence, the hippocampus has been implicated in the regulation of choice impulsivity per se as well as specific aspects of choice behavior.
The regulation of expected reward, however, is primarily associated with the NAc, as accumbal neurons have been activated by cues that signal potential rewards (Martin and Ono, 2000). Along with its dense, reciprocal connections with the BLA and hippocampus, as well as innervations from many other brain structures of the limbic corticostriatal loop, the NAc represents a key node in the control and modulation of reward-related aspects of behavior (Winstanley et al., 2006a; de Boer et al., 2017). Anatomically, the NAc can be divided into a dorsolateral core and a ventromedial shell region (Voorn et al., 2004), which appear to be differentially involved in regulatory mechanisms of impulsivity. As stated above, previous studies mainly found that NAc lesions increased impulsive choices in delayed reinforcement. This effect was particularly reported with inactivation of the AcbC. Consistently, performance in an adjusting-delay procedure induced neuronal activation in this region (da Costa Araújo et al., 2010). Thus, special attention has been paid to the AcbC, whereas the AcbSh has been studied less often, and these studies either reported increased choice behavior (Feja et al., 2014) or no effect following AcbSh lesions (Pothuizen et al., 2005). Hence, both subregions of the NAc appear to play a role in choice impulsivity, but with a supposedly greater involvement of the core subregion.
Altogether, previous literature provides evidence for anatomical and functional interactions within distinct pathways of the limbic corticostriatal loop in the regulation of choice impulsivity as well as its underlying functional mechanisms with regard to timing and reward evaluation (Winstanley et al., 2004b; Smith et al., 2015; Marshall and Kirkpatrick, 2016; Dalley and Ersche, 2019).
The current thesis aims to confirm and extend previous work on cortical and subcortical brain regions underlying choice impulsivity by focusing on neuronal activation in healthy individuals following delay-discounting performance as opposed to lesion-induced or pharmacological manipulations.

1.6.4. The mesencephalic dopaminergic system

In the central nervous system, the main output source of the neurotransmitter dopamine are the three mesencephalic cell groups VTA, SNc and retrorubral field (RRF) (Björklund and Dunnett, 2007a). The VTA is located in the ventral midbrain, medially adjacent to the substantia nigra and rostrally adjacent to the RRF. Dopaminergic innervations from the VTA form the mesocortical pathway by projecting to the mPFC, and the mesolimbic pathway which reaches limbic structures such as amygdala, hippocampus and NAc.

Nigrostriatal pathways are formed by dopaminergic neurons of the SNc which mainly reach the dorsal striatum and contribute to the control of voluntary movements, but which additionally connect to the ventral striatum, i.e., the NAc (Zeiss, 2005; Lammel et al., 2008).

Unlike dopaminergic innervations arising in the VTA, contributions of nigrostriatal connections to impulsive choice are inconclusive. Lesions of the SNc, on the one hand, were reported to have no effect on choice behavior (Magnard et al., 2018), while lesions of its main projection site, the dorsal striatum, increased choice impulsivity in rats (Tedford et al., 2015). Inactivations of these brain regions, however, often also impaired motor function and thus made it difficult to distinguish these effects from behavioral changes in impulsivity (Tedford et al., 2015; Magnard et al., 2018).

By contrast, mesocortical and mesolimbic connections are essential elements of the reward processing circuit based markedly on the modulatory influence of dopamine (Floresco and Magyar, 2006; Beaulieu and Gainetdinov, 2011). This was, for example, shown by increased dopamine release in the AcbC after optogenetic stimulation of neurons in the VTA during delay-discounting performance (Saddoris et al., 2015). Moreover, the authors found that this stimulation specifically affected delay-based choice, whereas changes in reward size did not influence dopamine levels in the NAc (Saddoris et al., 2015).

The involvement of VTA neurons in choice behavior, however, is not only attributed to its projections. VTA neurons themselves were shown to increase their firing rate in response to delayed reinforcement as assessed by electrophysiological recordings (Roesch et al., 2007). Moreover, the latter study observed that neuronal responses were additionally associated with changes in reinforcer value as well as with reward-predicting stimuli. Hence, VTA neurons have been implicated in reward prediction errors, i.e., the discrepancy between expected and actual rewards, in the context of choice impulsivity as well as other forms of reward-based learning (Schultz et al., 1997; Matsumoto and Hikosaka, 2009; Glimcher, 2011; Cohen et al., 2012).

1.6.5. The dorsal raphe nucleus

The most caudally located brain region which is heavily involved in impulsive choice behavior is the dorsal raphe nucleus (DRN). The DRN is a major source of the neurotransmitter serotonin, which, along with the dopaminergic system, plays an important role in the control of impulsivity (Winstanley et al., 2006b).

Projections from the DRN are widely distributed in the brain and reach several cortex regions including the frontal cortex, as well as piriform and entorhinal cortices. The mPFC and OFC are, thereby, both reciprocally connected with the DRN via descending glutamatergic projections to the DRN and ascending serotonergic inputs to the frontal cortex regions (Celada et al., 2001; Soiza-Reilly and Commons, 2011; Ogawa et al., 2014; Muzerelle et al., 2016).

Subcortical areas innervated by the DRN include various amygdalar nuclei, especially BLA and CeA, as well as the NAc. In the diencephalon, the lateral habenula is heavily connected to the DRN, but inputs

from thalamic and hypothalamic subregions are found as well (Hoover and Vertes, 2007; Muzerelle et al., 2016; Ko, 2017; Ren et al., 2018; Babalian et al., 2019).
The DRN additionally has strong reciprocal connections with VTA and SNc, i.e., the midbrain dopaminergic system (Ogawa et al., 2014; Muzerelle et al., 2016), a connection thought to play an important modulatory role in reward networks. Dopaminergic input from the VTA and SNc is suggested to target dopamine receptors on DRN neurons, particularly receptors of the D2 subfamily, resulting in stimulation of these neurons and the initiation of reward processes (Haj-Dahmane, 2001; Nakamura, 2013; Miyazaki et al., 2014; Fonseca et al., 2015; McDannald, 2015).
The DRN was generally implicated in reward-related behaviors (for review see: Luo et al., 2015; Nakamura, 2013) such as reward expectation or waiting for delayed rewards, as demonstrated by optogenetic and electrophysiological studies (Miyazaki et al., 2014; Fonseca et al., 2015; Li et al., 2016). More precisely, optogenetic stimulation of serotonergic DRN neurons has shown that mice increased their ability to tolerate longer delays during the performance of a delayed-reward task (Miyazaki et al., 2014).
Hence, the DRN connects to other brain regions of the reward circuitry, such as OFC and NAc, with which it additionally shares functional similarities, especially regarding the processing of expected rewards.

Collectively, brain structures related to impulsive choice show a complex pattern of interconnectivity, and within this network, each brain region serves a specific purpose in processing of reward- or delay-based aspects of this behavior. Moreover, dopamine and serotonin add a neurochemical component to these neuronal circuits by acting as neuromodulators in the adjustment of impulse control (Eagle and Baunez, 2010; Winstanley, 2011; Dalley and Roiser, 2012).

1.7. Neuronal correlates of aggressive behavior

In rodents, several brain regions involved in impulsive choice behavior overlap with brain regions associated with aggression and these may interact in the regulation of impulsive forms of aggressive behavior (Winstanley et al., 2005).
Brain areas of the telencephalon that are involved in the regulation of aggression include the olfactory bulb (OB), which is the rostralmost part of the telencephalon, as well as mPFC and OFC. Subcortical structures encompass the amygdala, lateral septum (LS), Bed nucleus of the stria terminalis (BNST), NAc, hippocampus, and the lateral habenula. The hypothalamus of the diencephalon, and particularly the hypothalamic attack area (HAA), is a crucial brain region involved in attack control and stress responses. Additional components of the aggression network are the VTA, the DRN and the periaqueductal grey (PAG), a structure located in the brainstem, which is strongly connected to the HAA and also implicated in attack control (Veenema and Neumann, 2007; Ko, 2017; Aleyasin et al., 2018b). For an overview of brain regions involved in offensive aggression see figure 1.2.
The next sections will give an overview of a selection of these neuronal correlates of aggressive behavior in terms of connectivity, general function and specific involvement in territorial, inter-male aggression.

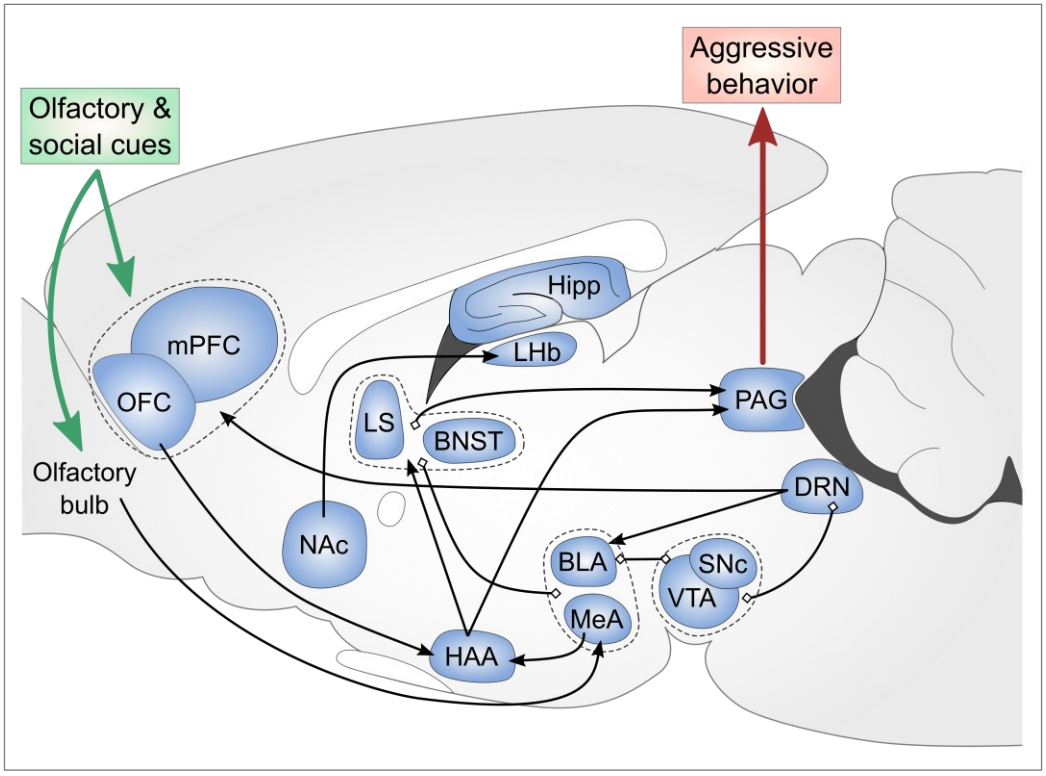

Figure 1.2 | Neuroanatomical pathways and structures involved in offensive aggression in rodents. The simplified schematic shows that important sensory stimuli (green arrow) are processed by the olfactory bulb and transmitted to the medial amygdala (MeA). To mediate species-typical aggression, information from the MeA is relayed to the lateral septum (LS) and the Bed nucleus of the stria terminalis (BNST), which contribute to the regulation of stress-related responses and the evaluation of social information. The hippocampus (Hipp) is another important area involved in the regulation of social stress or other emotional states linked to aggression, being connected to the hypothalamus, the midbrain dopaminergic system and the dorsal raphe nucleus (DRN) (not shown). The MeA also targets the hypothalamus, especially the hypothalamic attack area (HAA), and the periaqueductal grey (PAG) which are important for the initiation of attack behavior and the execution of aggression (red arrow). Connections from the frontal cortex, including orbitofrontal (OFC) and medial prefrontal (mPFC) areas, to the HAA or the amygdala are suggested to exert a modulatory influence on aggressive behavior. The nucleus accumbens (NAc) and the lateral habenula (LHb) appear to be involved in aggression-associated reward. The midbrain dopaminergic system, including substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), as well as the DRN within the brainstem additionally modulate aggressive behavior via dopaminergic and serotonergic inputs, respectively, to several of the aforementioned brain regions. Dashed lines denote closely related structures which are grouped for a simplified view of parallel projections. Line endings represented by an open square thereby indicate brain regions that share inputs from or outputs to the same areas. Lines with two squared endings represent reciprocal connections. BLA, basolateral amygdala. The presented graphic is based on findings reviewed by Aleyasin et al. (2018b), de Boer et al. (2017) and Nelson and Trainor (2007).

1.7.1. Sensory information and the amygdala

During inter-male confrontations, sensory stimuli, especially olfactory signals, are important for the assessment of social information of an opponent (Takahashi and Miczek, 2014). These social olfactory stimuli, including pheromones and other odorants of an intruder, are initially processed by the olfactory bulb and subsequently transmitted to the medial amygdala (MeA) (Sah et al., 2003; Nelson and Trainor, 2007; de Boer et al., 2015; Imamura et al., 2020).

Information from the MeA is further relayed to structures including LS, BNST, hypothalamic subregions, and PAG to mediate species-specific aggression (Nelson and Trainor, 2007; Ko, 2017; Aleyasin et al., 2018b).
In general, the amygdala is involved in the assessment of emotional values and emotional conditioning (O'Connell and Hofmann, 2011; Maeng and Shors, 2013; Nakamura, 2013).
Along with the MeA, the BLA has been identified as an important brain region in aggressive behavior. For both of these structures, studies on c-Fos induction have consistently found increased neuronal activation levels following aggressive acts in resident-intruder confrontations (Halász et al., 2002a; Veening et al., 2005; Veenema et al., 2007; Toth et al., 2012; Konoshenko et al., 2013; Hong et al., 2014; Biro et al., 2017). Moreover, Hong and colleagues were able to attribute the contribution of the MeA to inter-male aggression to specific neuron subpopulations, because optogenetic stimulation of GABAergic neurons reliably promoted offensive attacks, while glutamatergic neurons in the MeA inhibited attack behavior (Hong et al., 2014).
The MeA is additionally involved in other social behaviors, such as mating or parental care (Veening et al., 2005), and it was shown to contribute to maternal aggression as well (Unger et al., 2015).
The BLA was also shown to be involved in the evaluation of social interactions (Felix-Ortiz and Tye, 2014), and it is heavily implicated in fear and anxiety-like behavior, including conditioned fear and extinction learning (Duvarci and Pare, 2014). Moreover, in conjunction with serotonin, the BLA is thought to be an intersection point in modulatory processes of these behaviors, because it receives one of the densest serotonergic inputs within the amygdala, mainly originating in the DRN (Muzerelle et al., 2016; Linley et al., 2017). Glutamatergic projections from cortical areas additionally affect information processing in the BLA, especially innervations from frontal cortex regions, such as the mPFC, which have been implicated in the processing of conditioned responses (Sesack and Grace, 2010; Duvarci and Pare, 2014; Bocchio et al., 2016; Ko, 2017).

1.7.2. Frontal cortex regions

The frontal cortex, generally important for executive and cognitive functions (Winstanley et al., 2006a; Schoenbaum et al., 2011; Fitoussi et al., 2015), is suggested to play a modulatory role in aggressive behavior (Halász et al., 2006; Miczek et al., 2015).
Following inter-male confrontations, increased neuronal activation as indicated by c-Fos protein levels have been reported for several frontal cortex regions, including OFC and mPFC (Toth et al., 2012; Biro et al., 2017). Moreover, in the OFC, excitotoxic lesions were found to increase aggressive behavior in rats (Rudebeck et al., 2007).
A similar effect was observed for the mPFC in mice, as optogenetic inactivation of this region promoted aggression against a conspecific intruder (Takahashi et al., 2014). In addition, stimulation of the mPFC in the latter study correspondingly reduced aggressive behavior. These findings indicate an inhibitory role of the frontal cortex in inter-male territorial aggression.
With regard to corticolimbic pathways, the frontal cortex is assumed to communicate with the amygdala via a top-down modulatory influence to adapt aggression appropriately or maintain its expression within a normal range (Siever, 2008; Takahashi et al., 2014; Biro et al., 2017).

1.7.3. Lateral septum and Bed nucleus of the stria terminalis

Subcortical structures of the telencephalon which share connections with the amygdala and which are involved in aggression include the LS and the BNST.
The LS is located ventral to the corpus callosum at the midline, medially adjacent to the lateral ventricles. The BNST is located ventral to the septum, surrounding the anterior commissure and at its caudal end it lies dorsal to the hypothalamus (Paxinos and Watson, 1997).
The BNST is part of the extended amygdala which is involved in the regulation of stress responses and anxiety-like behaviors (Linley et al., 2017; Numa et al., 2019). The extended amygdala is a structure which resembles several nuclei of the amygdala proper with regard to connections, anatomical structure and functional contributions, depending on the specific subnuclei (for review see: Linley et al., 2017; Numa et al., 2019; Sah et al., 2003).
The expression of aggressive behavior during inter-male confrontations was shown to induce neuronal activation in LS and BNST as reported by c-Fos studies (Halász et al., 2002a; Veening et al., 2005; Veenema and Neumann, 2007; Toth et al., 2012; Konoshenko et al., 2013).
In addition to implications in aggression, the LS and BNST are both generally involved in social affiliative behaviors, such as social recognition or partner preference, and stress-related responses (Halász et al., 2002b; Lebow and Chen, 2016). All these behavioral aspects are closely associated with one another and contribute to the evaluation of social information and the initiation of appropriate actions during interactions with conspecifics (Lebow and Chen, 2016).
Within the information processing network for aggression, the LS, BNST, and MeA further project to subregions of the hypothalamus (Lebow and Chen, 2016; Hashikawa et al., 2017). It was, for example, shown that GABAergic projections from the LS modulate neuron activity in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl), a subregion of the hypothalamus crucially involved in attack control (Wong et al., 2016). In the latter study, lesions of the LS increased aggression in RI tests by disabling the innervations to the hypothalamus.

1.7.4. The hypothalamus

The hypothalamus, located in the ventral diencephalon, is a crucial brain region involved in the control of attack behavior (Veenema and Neumann, 2007), stress responses and sexual behavior (Hull et al., 2004; Veening et al., 2005). It comprises several subnuclei, including the so-called hypothalamic attack area (HAA), which is one of the most important areas for the regulation of aggression within the hypothalamus (Siegel et al., 1999; Hashikawa et al., 2017).
The subregions reported to comprise the HAA include the VMHvl, the anterior hypothalamic area, tuber cinereum and the lateral hypothalamus, all of which are anatomically adjacent to each other (Haller et al., 2006; Nelson and Trainor, 2007; Ko, 2017; Aleyasin et al., 2018b).
The HAA is crucial for the initiation of aggressive acts (for review see: Golden et al., 2019) as electrical or optogenetic stimulation of this region was shown to result in increased neuronal activation and the induction of offensive attacks when confronted with an intruder (Halász et al., 2002b; Lin et al., 2011). Halász and colleagues additionally observed that HAA stimulation induced c-Fos protein expression in interconnected brain regions, including LS, BNST and MeA, but also affected the paraventricular nucleus, PAG and Locus coeruleus (Halász et al., 2002b). These results are consistent with a series of other c-Fos induction studies implicating these brain regions in aggression control (Halász et al., 2002a; van der Vegt et al., 2003; Veening et al., 2005; Veenema et al., 2007; Toth et al., 2012; Konoshenko et al., 2013; Biro et al., 2017; Mark et al., 2019).

1.7.5. Midbrain and brainstem structures

Within the midbrain and the brainstem, structures that have been implicated in aggression include VTA, DRN and PAG.
Involvement of the midbrain dopaminergic system was shown by optogenetic stimulation of VTA neurons in mice which resulted in increased aggressive behavior against an intruder (Yu et al., 2014).
In the case of the DRN, an increase in territorial aggression was reported by Niederkofler et al. (2016) who inhibited the release of serotonin using genetic silencing. Additionally, aggressive acts during inter-male confrontations were found to induce neuronal activation in the DRN (van der Vegt et al., 2003; Mark et al., 2019), although another study found no changes in c-Fos expression levels (Toth et al., 2012). The VTA and the DRN thus appear to modulate aggressive behavior via their respective dopaminergic and serotonergic signaling (for details see sections 1.8. and 1.9.).
Investigations on c-Fos protein expression in the PAG of rats and hamsters have as well found increased neuronal activation following resident-intruder confrontations (Delville et al., 2000; Halász et al., 2002a; Veening et al., 2005), also in the case of rodent models of escalated aggression (Haller et al., 2006; Veenema and Neumann, 2007; Konoshenko et al., 2013; Tulogdi et al., 2015). Moreover, early studies performed in cats demonstrated that electrical and chemical stimulation of the PAG can elicit attacks and defensive rage (for review see: Siegel et al., 1999). Thus, the PAG has been associated with various forms of aggression, including territorial, predatory and abnormal aggression.
In this regard, the PAG receives inputs from the amygdala and hypothalamus and it projects to autonomic regions of the brainstem to regulate physiological responses in the context of agonistic confrontations (Siegel et al., 1999; Nelson and Trainor, 2007; Koolhaas et al., 2010a). Hence, the PAG represents an important output structure for aggressive behavior (Halász et al., 2002b; Nelson and Trainor, 2007).

1.8. Serotonin

Since its discovery in the early 1950s, the monoaminergic neurotransmitter serotonin, or 5-Hydroxytrypamine (5-HT), is one of the most extensively studied signaling molecules in the central nervous system (Nichols and Nichols, 2008; Olivier, 2015).
Its distribution is widespread in the brain and therefore it is not surprising that its functional contribution is very diverse as well. It has been implicated in cognitive processes, learning and memory, emotional control, as well as physiological functions such as stress, sleep or appetite (for review see: Carhart-Harris and Nutt, 2017; Nichols and Nichols, 2008). Serotonin has also been associated with the control and modulation of behavioral functions including impulsivity and aggression (for review see: Bortolato et al., 2013; Grigoryan, 2012; Nakamura, 2013).

1.8.1. Serotonergic neurotransmission

Serotonin is a neurotransmitter that is mainly synthesized in the dorsal and median raphe nuclei in the brainstem (Grigoryan, 2012; Nakamura, 2013). Effects of serotonin are mediated via 14 receptor subtypes from seven receptor families (5-HT_{1-7}), of which six families are transmembrane proteins coupled to G-proteins, while 5-HT_3 is an ionotropic receptor and exerts its action via a ligand-gated ion channel (Barnes and Sharp, 1999; Millan et al., 2008). In general, the coupling to stimulating

Table 1.1 | **Effects on choice impulsivity by systemic and local administration of serotonin receptor compounds in rodents**

Drug \ Administration form	Systemic administration		Local administration	
5-HT$_{1A}$ agonist				
8-OH-DPAT	↑[#]	(Evenden and Ryan, 1999)	mPFC =	(Yates et al., 2014)*
	↑[#]	(Winstanley et al., 2005)	OFC ↓	(Yates et al., 2014)*
	↑	(Stanis et al., 2008)		
	↑	(Blasio et al., 2012)*		
	↓	(Bizot et al., 1999)**		
	↓	(Zaichenko et al., 2013)		
	=	(Mori et al., 2018)		
Flesinoxan	↑[#]	(van den Bergh et al., 2006a)		
5-HT$_{1A}$ antagonist				
WAY 100635	=	(Evenden and Ryan, 1999)	mPFC =	(Yates et al., 2014)*
	=	(Winstanley et al., 2005)	OFC =	(Yates et al., 2014)*
	=	(Zaichenko et al., 2013)		
	=	(Liu et al., 2004)		
	↑[##]	(Bizot et al., 1999)**		
5-HT$_{1A/1B}$ agonist				
Eltoprazine	↓	(Korte et al., 2017)		
	↓	(van den Bergh et al., 2006a)		
5-HT$_{1B/1D}$ antagonist				
GR-127935	=	(van den Bergh et al., 2006a)		
5-HT$_{2A/C}$ agonist				
DOI	↑	(Evenden and Ryan, 1999)	mPFC =	(Yates et al., 2014)*
	↑	(Blasio et al., 2012)*	OFC =	(Yates et al., 2014)*
			OFC ↑	(Wischhof et al., 2011)**
5-HT$_{2A/C}$ antagonist				
Ketanserin	=	(Talpos et al., 2006)	mPFC =	(Yates et al., 2014)*
	=	(Paterson et al., 2012)	OFC =	(Yates et al., 2014)*
Ritanserin	=	(Evenden and Ryan, 1999)		
5-HT$_{2B/C}$ antagonist				
SER-082	↓	(Talpos et al., 2006)		
5-HT$_{2C}$ antagonist				
SB-242084	↓	(Paterson et al., 2012)		
5-HT$_{3}$ antagonist				
MDL-72222	=	(Evenden and Ryan, 1999)		
Granisetron	↓	(Mori et al., 2018)		
Ondansetron	↓	(Mori et al., 2018)		
5-HT$_{6}$ antagonist				
SB-270146-A	=	(Talpos et al., 2006)		

Note. ↑ increased impulsive choice, ↓ decreased impulsive choice, = no effect; mPFC, medial prefrontal cortex; OFC, orbitofrontal cortex; NAc, nucleus accumbens; *subjects tested in adjusting-amount procedure; **subjects tested in a T-maze; [#]decrease at 0-s delay or short delays; [##]dose-dependent effect

G-proteins (G_s, $G_{q/11}$) activates intracellular signaling pathways which activate second messengers, such as adenylyl cyclase or phospholipase C, and which finally stimulates neuronal signaling. Serotonin receptor subtypes that fall into this group include the 5-HT_2, 5-HT_4, 5-HT_6 and 5-HT_7 families. In contrast, receptors can be negatively coupled to adenylyl cyclase via other types of G-proteins ($G_{i/0}$) and thus inhibit neuronal signaling. The 5-HT_1 and the 5-HT_5 are such receptor families (Barnes and Sharp, 1999; Millan et al., 2008).

The majority of serotonin receptors are expressed as postsynaptic heteroreceptors at serotonergic projection sites such as the frontal and entorhinal cortices, striatum, hippocampus, hypothalamus and amygdala (for review see: Mengod et al., 2015; Millan et al., 2008; Olivier, 2004). Certain serotonin receptor subtypes additionally act as somatodendritic autoreceptors on serotonin neurons and presynaptic autoreceptors at serotonergic nerve terminals where they regulate serotonergic neuron firing and serotonin release. These receptors are primarily located in raphe nuclei or the frontal cortex and comprise the A, B and D subtypes of the 5-HT_1 family (Stamford et al., 2000; Mengod et al., 2015). Involvement of serotonin receptors in impulsive choice behavior and territorial aggression were mainly reported for the 5-HT_{1A}, 5-HT_{1B} and 5-$HT_{2A/C}$ subtypes, as demonstrated by pharmacological investigations (for review see: Bortolato et al., 2013; Jupp and Dalley, 2014; Takahashi et al., 2011; Winstanley, 2011). The findings from these earlier studies are summarized in tables 1.1 and 1.2 to provide detailed information on the effects of serotonin receptor compounds on impulsive choice and territorial aggression in rodents.

In the current thesis, the 5-HT_{1A} receptor is selected for further investigation and thus the following section more precisely illustrates its association with choice impulsivity and inter-male aggression.

1.8.2. The 5-HT_{1A} receptor in aggression and choice impulsivity

The relation between serotonergic neurotransmission, aggression and impulsivity was initially established on the basis of clinical studies in human subjects. In particular, a reduction in serotonin levels or its metabolites were associated with impulsive tendencies and enhanced aggressive behavior in these patients which was subsequently termed the serotonin deficiency hypothesis (for review see: Coccaro et al., 2015). However, in humans as well as rodents, this association was primarily reported for abnormal or violent forms of aggression (de Boer and Koolhaas, 2005; Mosienko et al., 2012; Coccaro et al., 2015). By contrast, adaptive aggression, including inter-male confrontations typically assessed in resident-intruder tests, has been positively linked to serotonin expression (de Boer et al., 2009) and increased serotoninergic neurotransmission (Mark et al., 2019).

Especially the 5-HT_{1A} receptor has been attributed an anti-aggressive effect, because 5-HT_{1A} receptor agonists consistently decreased species-typical aggressive behavior in inter-male confrontations (Olivier et al., 1995; de Boer et al., 2000; de Boer and Koolhaas, 2005). It was suggested that this effect is specifically mediated via 5-HT_{1A} autoreceptors in the DRN due to a reduction in serotonin release within its target areas (Koolhaas et al., 2010b). This notion is supported by the observation that injection of 5-HT_{1A} agonists locally into the DRN also reduced offensive aggression in RI tests (Mos et al., 1993; van der Vegt et al., 2003). However, a more recent study by D. J. Stein et al. (2013) which investigated pharmacological effects within subregions of the frontal cortex appears to contradict these findings. Local agonism of 5-HT_{1A} in the OFC reduced aggression by presumably acting on postsynaptic heteroreceptors which inhibit the activity of non-serotonergic neurons in this region. This corresponds to an increase in serotonin signaling rather than a reduction caused by autoreceptor activation. Within the mPFC, however, the authors found no effect on aggression (D. J. Stein et al., 2013), although the 5-HT_{1A} subtype is highly expressed in several frontal cortex regions (Aznar et al., 2003; Santana et al., 2004).

Hence, the modulation of territorial aggression mediated by 5-HT_{1A} receptors appears to depend on the cellular localization of this receptor at the pre- or postsynapse, and on its expression within different brain regions.

Similar dependencies on 5-HT_{1A} localization are as well indicated for choice impulsivity, but additional factors seem to influence this behavior in delayed-reward tasks, such as the applied paradigm or the dose of the used receptor compound. However, the precise role of 5-HT_{1A} in the control and modulation of choice impulsivity remains to be clarified.

As an initial step to characterize the relation between serotonin and choice behavior, early pharmacological studies in rodents investigated systemic serotonin depletion by use of the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT).

Table 1.2 | **Effects on territorial aggression by systemic and local administration of serotonin receptor compounds in rodents**

Drug \ Administration form	Systemic		Local	
5-HT$_{1A}$ agonist				
8-OH-DPAT	↓*	(Olivier et al., 1995)	DRN ↓	(Mos et al., 1993)
	↓	(de Boer et al., 2000)		
Alnespirone	↓	(de Boer and Koolhaas, 2005)	DRN ↓	(van der Vegt et al., 2003)
S-15535	↓	(de Boer et al., 2000)*		
	↓	(de Boer and Koolhaas, 2005)		
F-15599			OFC ↓	(D. J. Stein et al., 2013)
			mPFC (IL) =	(D. J. Stein et al., 2013)
5-HT$_{1A}$ antagonist				
WAY-100635	=	(de Boer et al., 2000)	OFC =	(D. J. Stein et al., 2013)
	=	(de Boer and Koolhaas, 2005)		
WAY-100135	↓#	(Bell et al., 1996)		
	↑#	(Bell et al., 1996)		
5-HT$_{1A/1B}$ agonist				
Eltoprazine	↓	(Olivier et al., 1995)	DRN ↓	(Mos et al., 1993)
	↓	(de Boer and Koolhaas, 2005)		
5-HT$_{1B}$ agonist				
Anpirtoline	↓	(de Almeida and Miczek, 2002)		
CGS-12066B	↓	(de Boer and Koolhaas, 2005)		
CP-93129	↓	(de Boer and Koolhaas, 2005)		
5-HT$_{1B/1D}$ antagonist				
GR-127935	=	(de Almeida and Miczek, 2002)		
	=	(de Boer and Koolhaas, 2005)		
5-HT$_{2A/C}$ agonist				
DOI	↓	(Olivier et al., 1995)		

Note. ↑ increased territorial aggression, ↓ decreased territorial aggression, = no effect; DRN, dorsal raphe nucleus; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NAc, Nucleus accumbens; OFC, orbitofrontal cortex; *non-specific anti-aggressive effect (simultaneous decrease in social interest); #dose-dependent effect

These studies mostly reported increased impulsivity levels (Wogar et al., 1993; Bizot et al., 1999; Mobini et al., 2000), being in accordance with the serotonin deficiency hypothesis, although others did not find any effect on choice behavior in response to 5,7-DHT (Winstanley et al., 2003, 2004a).
Contradictory results have also been reported for selective 5-HT_{1A} receptor compounds. While systemic administration of the 5-HT_{1A} antagonist WAY 100635 only affected choice behavior in one study (Bizot et al., 1999), local application showed no effect (Yates et al., 2014). By contrast, systemic agonism of this receptor dose-dependently either increased (Evenden and Ryan, 1999; Winstanley et al., 2005; Stanis et al., 2008; Blasio et al., 2012) or decreased choice behavior (Bizot et al., 1999; Zaichenko et al., 2013). Moreover, decreased impulsive choices were found with local injections into the OFC, but not into the mPFC (Yates et al., 2014).
Therefore, one objective of the present thesis is to investigate the expression of the 5-HT_{1A} receptor in individuals with varying levels of impulsivity and aggression, not only in the frontal cortex, but within several brain areas underlying these behaviors.

1.9. Dopamine

Dopamine is the quantitatively most abundant monoaminergic neurotransmitter in the brain. It mediates various functions including motivational processes, motor performance, stress responses, learning and multiple other cognitive mechanisms (Björklund and Dunnett, 2007a; Beaulieu and Gainetdinov, 2011). Dopamine is especially known for its role in the regulation of reward and thus it has long been associated with impulsive behavior (Dalley and Roiser, 2012).

1.9.1. Dopaminergic neurotransmission

The neurotransmitter dopamine is mainly synthesized in three cell groups localized in the ventral midbrain: the retrorubral field (A8 cell group), the substantia nigra pars compacta (A9; SNc) and the ventral tegmental area (A10; VTA) (Vitalis et al., 2005; Björklund and Dunnett, 2007a). Dopamine exerts its effect by binding to one of five transmembrane receptors which are classified into D1- and D2-like receptor families according to their interaction with different G-proteins (Beaulieu and Gainetdinov, 2011).
The D1-like family comprises D1 and D5 receptors which are expressed postsynaptically on non-dopaminergic neurons and which stimulate adenylyl cyclase via G_S-protein coupling and thus promote neuronal signaling (Beaulieu and Gainetdinov, 2011; Ford, 2014). Within the D1-like family, expression levels of the D1 receptor subtype are higher compared to the D5 receptor, and D1 is found in all major dopaminergic pathways (mesocortical, nigrostriatal, mesolimbic), including frontal cortex regions, nucleus accumbens, dorsal striatum, substantia nigra and amygdala (Beaulieu and Gainetdinov, 2011).
The D2-like receptor family implies D2, D3 and D4 receptors which are coupled to inhibitory G-proteins ($G_{i/o}$) and thus reduce neuronal signaling (Beaulieu and Gainetdinov, 2011; Ford, 2014). These receptor subtypes are found at dopaminergic projection sites such as the frontal cortex, striatum or limbic areas like the amygdala and the hippocampus (Richtand et al., 2010; Beaulieu and Gainetdinov, 2011). Dopaminergic receptors, however, are also found at their sites of synthesis at which the D2 receptor is known to act as presynaptic autoreceptor on dopaminergic neurons (Ford, 2014; Gallo, 2019). In contrast to heteroreceptor function, the activation of D2 autoreceptors leads to inhibitory feedback mechanisms which can reduce DA synthesis, DA release and increase DA reuptake (for review see: Zhang and Sulzer, 2012). An autoreceptor function was also suggested for the D3 receptor, but previous investigations remain inconclusive (e.g., Koeltzow et al., 1998; Richtand et al., 2010).

Moreover, within the D2-like family, the D2 receptor is the most abundant subtype, whereas the D4 receptor shows the lowest expression levels in the brain (Richtand et al., 2010; Beaulieu and Gainetdinov, 2011). With regard to choice impulsivity, D4 and D5 receptors were only scarcely investigated (Koffarnus et al., 2011; Meda et al., 2019). Previous literature has largely focused on the D1 and D2 subtypes, due to their widespread distribution and high expression levels, and because of their implication in a variety of functions including the regulation of impulsive behavior (Jupp and Dalley, 2014; Yates and Bardo, 2017).

1.9.2. The dopaminergic system and impulsive choice

The role of dopaminergic neurotransmission in impulsive choice was largely studied by pharmacological approaches using substances that manipulate dopamine release, reuptake or act on its receptors.

Choice impulsivity was found to be affected by compounds such as amphetamine, an indirect dopamine agonist, or the selective dopamine reuptake inhibitor GBR 12909. In a majority of studies both compounds reduced impulsive choice in delayed reward tasks (Wade et al., 2000; van Gaalen et al., 2006; Baarendse and Vanderschuren, 2012), although treatment with amphetamine has also been shown to increase choice impulsivity (Evenden and Ryan, 1996; Zeeb et al., 2016) or have no effect (Koffarnus et al., 2011). Both substances induce blockade of dopamine transporters and lead to an increase in dopamine release (Baarendse and Vanderschuren, 2012; Korte et al., 2017). Therefore, involvement of the dopaminergic system in impulsive decision-making was suggested to potentially underlie increased dopamine signaling (Baarendse and Vanderschuren, 2012; Korte et al., 2017).

The availability of more selective agents facilitated investigations on dopaminergic modulation of impulsive behavior, especially with focus on individual dopamine receptor subtypes (for review see: Jupp and Dalley, 2014; Winstanley, 2011). The findings from these earlier studies are summarized in table 1.3 to provide detailed information on the effects of dopamine receptor compounds on choice impulsivity in rodents.

Administration of D1-like antagonists, for example, was mainly reported to increase impulsive choice in delay-discounting paradigms when given systemically (van Gaalen et al., 2006; Koffarnus et al., 2011; Pattij et al., 2014; Li et al., 2015; Tian et al., 2019). A similar effect was reported for a D2-like antagonist in an adjusting-amount procedure (Wade et al., 2000), whereas most antagonists that target D2 receptors were shown to have no effect on the performance in delay-discounting tasks (Evenden and Ryan, 1996; van Gaalen et al., 2006; Koffarnus et al., 2011; Pattij et al., 2014; Li et al., 2015; Tian et al., 2019). These findings might suggest a greater role for D1-like receptors in the control of choice behavior compared to D2-like receptors.

However, investigations on local application of dopaminergic agents into specific brain regions yielded different results. In particular, following intra-mPFC infusions, the D1 antagonist SCH 23390 increased choice impulsivity similar to systemic administration (Loos et al., 2010; Pardey et al., 2013), but such an increase was also observed with use of a D1 agonist (Loos et al., 2010), a D2 agonist (Yates et al., 2014) and different D2 antagonists (Pardey et al., 2013; Yates et al., 2014). These findings implicate both receptor families in choice impulsivity and additionally highlight the mPFC as crucial brain region in the regulation of choice behavior. Furthermore, these contradictory results on D1 and D2 receptor compounds demonstrate the complex impact of these receptors on impulsive decision-making.

Table 1.3 | Effects on choice impulsivity by systemic and local administration of dopamine receptor compounds in rodents

Drug \ Administration form	Systemic	Local
D1 agonist		
SKF 81297	= (Koffarnus et al., 2011)	mPFC = (Yates et al., 2014)** OFC = (Yates et al., 2014)**
SKF 38393		mPFC ↑ (Loos et al., 2010) NAc = (Yates and Bardo, 2017)
D1 antagonist		
SCH 23390	= (Wade et al., 2000)* ↑ (van Gaalen et al., 2006) ↑ (Koffarnus et al., 2011) ↑ (Pattij et al., 2014) ↑ (Li et al., 2015) ↑ (Tian et al., 2019)	mPFC = (Yates et al., 2014)** mPFC ↑ (Loos et al., 2010) mPFC ↑ (Pardey et al., 2013) OFC = (Pardey et al., 2013) OFC = (Yates et al., 2014)** OFC = (Zeeb et al., 2010)
D1/D2 mixed antagonist		
Flupenthixol	↑ (Cardinal et al., 2000) ↑ (Wade et al., 2000) ↑ (Floresco et al., 2008)	
D2 agonist		
Sumanirole	= (Koffarnus et al., 2011)	
Quinpirole		mPFC ↑ (Yates et al., 2014)** OFC = (Yates et al., 2014)** NAc = (Yates and Bardo, 2017)
D2 antagonist		
Eticlopride	= (Li et al., 2015) = (van Gaalen et al., 2006) = (Pattij et al., 2014)	mPFC ↑ (Yates et al., 2014)** OFC ↑ (Zeeb et al., 2010)# OFC = (Yates et al., 2014)** NAc = (Yates and Bardo, 2017)
Raclopride	= (Tian et al., 2019) ↑ (Wade et al., 2000)*	mPFC ↑ (Pardey et al., 2013) OFC ↑ (Pardey et al., 2013)
Haloperidol	= (Koffarnus et al., 2011) = (Evenden and Ryan, 1996)	
L741,626	= (Koffarnus et al., 2011)	
D3 agonist		
7-OH-DPAT	↓ (van den Bergh et al., 2006a)	
Pramipexole (mixed D2/D3)	= (Koffarnus et al., 2011)	
D4 partial agonist		
ABT-724	↑ (Koffarnus et al., 2011)	
D4 antagonist		
L745,870	= (Koffarnus et al., 2011)	

Note. ↑ increased impulsive choice, ↓ decreased impulsive choice, = no effect; mPFC, medial prefrontal cortex; NAc, Nucleus accumbens; OFC, orbitofrontal cortex; *subjects tested in an adjusting-amount procedure; **subjects tested in an adjusting-delay procedure; #effects observed when delay was cued

Chapter 2

Relationship between impulsive choice and territorial aggression in male Dark Agouti inbred rats

Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

2.1. Abstract

Impulsivity and aggression are behaviors expressed in various forms in everyday life situations and are often closely associated with one another. The nature of these relations, however, may depend on specific behavioral aspects. In the present study, investigations on choice impulsivity and territorial aggression were conducted in male Dark Agouti (DA) inbred rats using a delay-discounting (DD) task and a resident-intruder (RI) test. Additionally, rats either performed the DD task prior or subsequent to RI tests to examine potential effects of test order. Overall, DA rats showed a high range of individual impulsive choice behavior and were classified as low, medium or highly impulsive, based on the preference for a delayed reward. Individual differences in choice impulsivity are mostly detected in outbred strains, and DA rats seem to represent an exception to this rule. The aggression range of DA rats was limited to low and medium levels, and at the same time, medium-aggressive rats spent less time in non-social behaviors than their low-aggressive counterparts. In this regard, behavioral profiles of DA rats differ compared to outbred or wild-type strains. An overall correlation for impulsivity and aggression was not observed in DA rats, but sensitivity to reinforcer magnitude and number of attacks were positively correlated. Changes in delay tolerance were only observed in rats that performed aggression testing prior to impulsivity testing. These differential contributions of reinforcer magnitude and delay to impulsive choice together with individual experiences in inter-male confrontations demonstrate the diversity of impulsive-aggressive manifestations in rodents.

2.2. Introduction

Impulsivity is a behavioral construct that shows multidimensional manifestations within the natural scope of personality traits (Evenden, 1999; Dalley et al., 2011). Maladaptations or deficient mechanisms of impulsive behavior or self-control also play a role in a variety of human personality disorders including addictions, gambling disorders or attention deficit/hyperactivity disorder (ADHD) (Winstanley, 2011; Robbins and Dalley, 2017).

Aspects of impulsivity incorporate unplanned or risky behavior with regard to outcome expectancy and an overall tendency to act without forethought as well as the inability to inhibit motor responses (Evenden, 1999; Dalley and Roiser, 2012; Rosell and Siever, 2015; Smith et al., 2015). This concept can be further partitioned into impulsive choice and impulsive action (Winstanley et al., 2006a; Dalley and Roiser, 2012).

Choice impulsivity is characterized by intolerance towards delays, while impulsive action implies mistimed response behavior (Dalley and Roiser, 2012). Impulsive choice occurs when two reward options differ in magnitude and time point of receipt. In rodents, it is classically assessed using delay-discounting (DD) paradigms which require the individual to decide between a small, immediate reward and a large, delayed reward (Evenden and Ryan, 1996). Impulsive individuals, hereby, devalue the large

reward with increasing delay time, irrespective of the rewards relative value, and often prefer the immediate choice option (Odum, 2011; Robbins and Dalley, 2017).

A behavioral trait often associated with impulsivity is aggression, which in humans is commonly classified into instrumental (proactive and premeditated) and impulsive aggression (reactive and hostile) (Cervantes and Delville, 2007; Siever, 2008; Neumann et al., 2010; Rosell and Siever, 2015; Wrangham, 2018). In general, aggression is an agonistic behavior directed against oneself or another person with the purpose to harm this individual (Nelson and Trainor, 2007). In rodents, male individuals often engage in aggressive acts when defending their territory to protect mating partners and to retain food sources (Takahashi and Miczek, 2014). Under laboratory conditions, the assessment of territorial aggression is performed by use of resident-intruder (RI) tests, in which the resident of an established territory is confronted with a male conspecific intruding this territory (Koolhaas et al., 2013).

It is often reported that heightened trait aggression is associated with heightened trait impulsivity (van den Bergh et al., 2006b; Cervantes and Delville, 2007; Nelson and Trainor, 2007), but relations between both appear to be more complex. Impulsive choice and action are facets of impulsivity which are widely considered two distinct entities with only partial overlap with regard to behavior, neuronal circuits and neurochemical basis (Winstanley et al., 2004a; Dalley et al., 2011; Nautiyal et al., 2017). When relationships of impulsivity and aggression are investigated, a distinction between both impulsivity aspects should be taken into account.

Positive correlations of aggression with impulsive choice were previously reported for Wistar Han rats (van den Bergh et al., 2006b) and golden hamsters (Cervantes and Delville, 2007), supporting the general theory of concurrent increase in both behaviors. In a rat line selectively bred for response in a novel environment, however, the high responder type of rat displayed less aggressive behavior together with heightened impulsive choice, whereas impulsive action increased with increasing aggression (Flagel et al., 2010; Kerman et al., 2011). A positive relation between aggression and impulsive action was also found in serotonin receptor knockout mice in both a go/no-go task (Nautiyal et al., 2015) and a differential reinforcement of low rates (DRL) task, during which a reward is earned only when a certain amount of time has passed between two lever presses (Evenden, 1999; Simon et al., 2013). Coppens et al. (2014) investigated response inhibition using a DRL task and found no relation between aggression and impulsivity in wild-type Groningen (WTG) rats. Violent individuals of the latter strain, however, displayed increased intolerance towards delays in the DRL task. In short, the type of impulsivity considered as well as the strain of rat seems to play a role whether or not an association of impulsivity and aggression exists.

Based on the divergent findings on relationships between different types of impulsivity and aggression, we addressed the relation between impulsive choice and territorial aggression in Dark Agouti (DA) inbred rats. We focused on choice impulsivity and investigated its individual manifestations as well as its relation to aggressive behavior performed in inter-male confrontations. Therefore, male DA rats underwent a DD procedure and a RI task. The DA inbred strain was chosen because previous observations in our laboratory showed that these rats displayed the whole spectrum of aggressive behavior from low to high as classified after de Boer and colleagues (2003) when confronted with Wistar rats (Radant, 2010). It is generally assumed that domestication and inbreeding narrows the phenotypic range of behaviors (Visscher et al., 2008; Koolhaas, 2010). However, in DA rats a normal distribution of aggressive behavior was reported (Radant, 2010). Therefore, we expected DA rats to display a variety of impulsive choice behavior including individuals with increased or decreased aversion towards delayed rewards in the DD task and to show a wide range of offensive aggressive behavior in inter-male confrontations, as reported earlier. Furthermore, with regard to previous

findings on impulsive-aggressive relationships, we expected that male DA rats show enhanced levels of aggression in confrontations with an intruder, when delay aversion in a DD task is increased as well. Additionally, we examined potential influences of one behavior on the other by using two test-order groups, which either performed the DD task prior or subsequent to the RI tests. Hereby, we expected similar results in both test-order groups for choice behavior as well as aggressive behavior, provided that both traits are stable over time.

2.3. Material and methods

2.3.1. Animals

A total of 32 male rats of the DA inbred strain (DA/OlaHsd) were used for behavioral testing. DA rats were commercially acquired (ENVIGO, Huntingdon, UK) and bred in our department. Male Wistar Han rats (n = 32) served as intruders in RI tests (Wistar Han IGS, Crl:WI (Han), Charles River, Sulzfeld, Germany; Wistar Han, RccHan^{TM}:WIST, Envigo). The Wistar strain was chosen as opponent in RI tests because it provokes increased aggression in DA rats (Radant, 2010) and as resident it shows a narrow aggressive profile limited to low- and medium-aggressive individuals (de Boer et al., 2003). Rats were kept under controlled climate conditions of 21 ± 2°C and 55 ± 5 % humidity on a 12 h light/dark cycle (lights on at 8:00 a.m.). DA and Wistar rats were kept in separate rooms; they were housed in groups of four to six individuals in Macrolon type IV cages with *ad libitum* water and food prior to testing. At the start of the operant-conditioning training, DA rats were eight to nine weeks old and weighed 180 g to 250 g. During operant training and the DD task, food was restricted, the weight was checked daily prior to behavioral testing and held constant between 85 % and 90 % of the free feeding weight of unrestricted rats. For RI tests, Wistar rats were food restricted to fall below (max. 18 %) or to match the weight of their DA counterparts. A radio was switched on for background noise in the animal-keeping facility during the light phase and in all test rooms during behavioral testing. In a previous study in our lab, DA rats performed the RI test during the night vs. the light phase and the duration of aggressive behavior as well as the number and latency of bites did not differ significantly (Radant, 2010). Hence, DD and RI tests were performed during the light phase. All experimental procedures were carried out in accordance with the German law on animal protection and the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health, 8th edition, 2011).

2.3.2. Experimental design

All DA rats underwent a DD paradigm to measure their impulsive choice behavior, and territorial, inter-male aggressive behavior was determined by use of RI tests. Prior to the DD task, rats completed an operant-conditioning training during which each DA rat received daily sessions of four training phases: Habituation, lever-press training, nose-poke training and large-reward baseline training. Subsequently, rats were randomly assigned to one of two test groups (n = 16 for each group). Rats of the group DD-RI performed the DD task directly after baseline training, followed by the RI test, while rats of the second test order group (RI-DD) performed the tests in a reversed order. For the latter group, an additional baseline training phase was included after completion of RI tests and prior to the DD task to ensure the maintenance of the large-reward preference (Fig. 2.1).

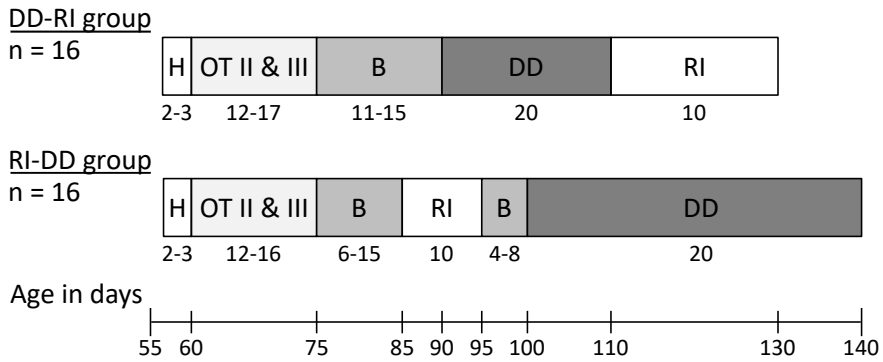

Figure 2.1 | **Experimental design and timeline for impulsivity and aggression testing of Dark Agouti rats.** Both test-order groups of rats are presented with the DD-RI test group in the upper panel and the RI-DD test group in the middle panel, both aligned to the age of the rats (lower panel). Operant training (OT) II & III correspond to lever-press and nose-poke training, respectively. Numbers below each training and test phase indicate the minimum and maximum number of daily sessions performed by the rats. B, large-reward baseline training; DD, delay-discounting task; H, habituation to test chamber; RI, resident-intruder test.

2.3.3. Operant-conditioning training and delay discounting

2.3.3.1. Apparatus

The test apparatus consisted of three operant-conditioning chambers (34 x 34 x 33 cm) each equipped with a house light, two retractable levers (ENV-112, Med Associates; or H23-17RB, Coulbourn Instruments, Holliston, United States) positioned five cm above a grid floor, and a food magazine situated between the levers and provided with an infrared beam and a magazine light. A pellet dispenser (ENV-203M-45, Med Associates) connected to the food magazine delivered the food reward (Dustless Precision Pellets®, 45 mg, Product# F0021, Bio-Serv, USA). Activation of the retractable levers was indicated by a green stimulus light above each lever. The operant-conditioning chambers were operated via a custom-built, computer-controlled panel and control software (Pascal-based, made in our lab).

2.3.3.2. Habituation

Initially, rats were familiarized with the pellets in their home cage and handled daily by the experimenter for 3-4 days. For habituation to the test chambers, rats were placed inside a chamber for 30 min with the house light illuminated and 20 pellets in the food magazine. When at least 70 % of the pellets per session were consumed on two consecutive sessions, lever-press training began.

2.3.3.3. Lever press

Lever-press training consisted of a continuous reinforcement schedule with only one lever presented and the house light illuminated during the whole session. Each lever press resulted in the delivery of one pellet, the illumination of the magazine light and the retraction of the lever. A session terminated either after 30 min or 100 lever presses. Right and left levers were trained successively, starting with the right lever. Lever-press training was successful with at least 50 % lever presses per session on each lever on three consecutive sessions.

2.3.3.4. Nose poke

After the association of the lever press with the food reward, rats learned to perform a nose poke into the food magazine to initiate the presentation of the levers. Nose poke sessions were structured into 100 discrete trials with fixed 30-s inter-trial intervals during which all lights were extinguished and levers were retracted. At the beginning of each trial, the house and magazine lights were illuminated and the rat had 10 s to perform a nose poke into the food magazine. A following lever press within 10 s resulted in the delivery of one pellet. If a nose poke or a lever press was not performed by the rat, the trial ended and was considered an omission. The presentation of left and right levers was counterbalanced within each session, with each lever presented not more than two times in sequence. Each trial ended either after the collection of the reward or after an omission. The session terminated either after 60 min, 60 successful trials or a maximum of 100 trials. Nose-poke training was successful at 80 % lever presses per session on three consecutive sessions.

2.3.3.5. Large-reward baseline

In the last training phase, each lever was assigned a different reward quantity: One pellet to a small-reward lever and three pellets a large-reward lever. The assignment of the large-reward lever was counterbalanced between rats and kept constant for each rat throughout the experiment. Each baseline session consisted of 60 trials divided into five blocks of 12 trials. Each block started with two forced-choice trials with either the left or right lever presented after nose poke. The order of forced-choice trials was pseudorandomized for each block. The following 10 trials were free-choice trials during which both levers were presented and the rat could choose between the small and the large reward. The choice for either lever resulted in the delivery of the corresponding reward and the retraction of both levers. Baseline sessions terminated after 60 min or 60 trials. As a precondition for the DD task, rats needed to establish a preference for the large reward. Three consecutive sessions with 80 % choices for the large reward were used as a criterion.

2.3.3.6. Delay-discounting task

For the assessment of impulsive choice, a within-session, increasing-delay procedure was chosen adapted from (Evenden and Ryan, 1996). Lever assignment and general procedure were the same as for the large-reward baseline training, but a delay to the large reward was introduced. Each rat completed 20 sessions which consisted of five blocks of 14 trials with increasing delays of 0, 10, 20, 40 and 60 s for blocks 1 to 5, respectively. The number of forced-choice trials was increased to four (2x left, 2x right) to illustrate timing conditions for each block to the rats. The inter-trial interval duration was chosen at a fixed 40 s, regardless of the rat's response, to compensate for the delay. Each session terminated either after the maximum of 70 trials or after 96 min.

2.3.4. Territorial aggression

The RI test was used for the examination of inter-male territorial aggression. Each DA rat was housed with a conspecific, oviduct-ligated female for six to seven days prior to testing to establish a territory. The home cage was equipped with new bedding and a wooden house, and *ad libitum* access to food and water was given. The bedding was not cleaned during the test phase to maintain the scent marks of the resident. For habituation, the resident was daily exposed to the following conditions on the last three days before testing: The female rat, house, food and water were removed from the cage, and

the cage was placed into the test room for 30 min under dimmed light conditions (150 lux). RI tests were performed on four consecutive days for each resident. The resident cage was positioned in the test room as described for habituation. After 30 min, an unfamiliar Wistar rat was placed into the cage for 10 min. The residents' behavior was recorded with a video camera for subsequent analysis and the experimenter remained in the room to monitor the test. After the intruder rat was removed from the cage, it was checked for injuries and placed back into its home cage. During the test phase, the resident was kept with its female companion. For each test, the resident was confronted with a different Wistar rat and each Wistar served as an intruder up to four times.

2.3.5. Statistical and behavioral analyses

Statistical analyses were carried out by use of SPSS for Windows (version 26.0). The significance level was set at $p < 0.05$ for all data analyses. Normal distribution was checked with the Shapiro-Wilk normality test and homogeneity of variance for repeated measures and mixed-model analysis of variance (ANOVA) was checked with Mauchly's test of sphericity. When the normality requirement was not met, data was rank transformed with mean rank for tied values. Violations of the sphericity requirement were corrected using the Greenhouse-Geisser estimate of sphericity (ε).

2.3.5.1. Impulsive choice behavior

For impulsive choice behavior, the last five DD sessions were analyzed for number of large-reward choices, percent of omitted trials, area under curve (AUC) values (Myerson et al., 2001) and *k* and A (y-intercept) values derived from a hyperbolic discounting function fitted to the data.

Particularly, large-reward choices for each delay were used to determine stable choice of individuals by repeated measures ANOVAs with session as within-subjects factor. Data for each delay within a session were treated as independent replicates. DD curves of individuals were computed with the number of large-reward choices plotted against delays. From these discounting curves, normalized AUC values were determined by taking the sum of the area under the curve divided by the maximal area of 600 s, thus, reaching values between 0 and 1. AUC values were used for classification of impulsive choice behavior by subdivision of the AUC value range into thirds. The impulsivity level is inversely proportional to AUC values, such that high AUC values correspond to a low level of choice impulsivity and vice versa. Hence, rats with AUC values in the upper, middle or lower third were classified as low (L-Imp), medium (M-Imp) or highly impulsive (H-Imp), respectively.

After classification, data on large-reward choices of the three impulsivity groups were pooled and underwent a group comparison. A mixed-model ANOVA was conducted with session and delay as within-subjects factors and impulsivity group as between-subjects factor. An overall significant ANOVA was followed by multiple comparisons using Bonferroni correction.

With regard to the large-reward data acquired for the 0-s delay, a decrease in preference was observed for the H-Imp group of rats which dropped below 80 %, and thus below the set success criterion for the large-reward preference. To identify group differences for this first session block, a non-parametric Kruskal-Wallis test with impulsivity group as a factor was conducted. This was followed by multiple comparisons using Bonferroni correction. Data analyses for the remaining four delay blocks are included in the mixed-model ANOVAs, due to the time dependency of these data.

Large-reward data was additionally analyzed using non-linear curve fitting. A hyperbolic discounting function, defined by the equation $V = A/(1+kD)$ (Mazur, 1987), was fitted to each individual's preference data using GraphPad Prism software (version 5.00). In the hyperbolic discounting function,

V is the subjective reinforcer value, A is the reinforcer amount, D is the delay to the large reward and *k* is a free parameter that corresponds to the slope of the preference curve illustrating the degree of discounting. For the curve fitting procedure, *k* and A parameters were chosen as free parameters and used as estimates for sensitivity to delay and sensitivity to reinforcer amount, respectively. The current approach was based on previously described procedures (Aparicio et al., 2015; Bezzina et al., 2008; Valencia-Torres et al., 2012) and earlier findings that both of these parameters can affect impulsive behavior independently (also see section 2.4.1.; Ho et al., 1999; Pitts and Febbo, 2004). High *k* values, i.e., a steep discounting curve, thereby, represent high choice impulsivity due to increased discounting in response to an increasing delay. Estimates of the A parameter correspond to the y-intercept of the discounting curve and represent an estimate for the rats' sensitivity to reinforcer magnitude when no delay is present (0-s delay). Subsequently, the three impulsivity parameters AUC, *k* and A underwent a correlation analysis using one-tailed Spearman's rank correlations.

2.3.5.2. Aggression

The behavior of each resident from all four RI tests was scored using the Behavioral Observation Research Interactive Software (BORIS, version 2.981) (Friard and Gamba, 2016). The videos were additionally analyzed by a second experimenter experienced in behavioral scoring. The inter-rater reliability for both observers showed a high degree of agreement with intra-class correlation coefficients between 0.89-0.99; therefore, a subset of videos was analyzed in duplicate. The total duration of the following measures was assessed and classified into four main behavioral categories: Offensive aggression (bite and clinch attacks, lateral threat, chase, keep down, offensive upright), social investigations (social exploration, mounting), non-social behaviors (cage exploration, rearing, inactivity) and grooming**.** Additionally, attack number and attack latency were determined. Rats were classified into aggression groups by mean time spent in offensive aggression in all four RI tests. The borders of classification were based on a study by de Boer and colleagues (2003) defining three aggression score classes in wild-type rats with borders at 15 % for low- (L-Agg) and medium-aggressive (M-Agg) rats and 55 % for M-Agg and highly-aggressive (H-Agg) rats. For each behavioral category, data for aggression groups was compared by use of Mann-Whitney U tests. Additionally, the relationship between aggression measures was assessed with one-tailed Spearman's rank correlations.

2.3.5.3. Correlations between behavioral measures

The relationship between impulsive choice behavior and behavioral measures from RI tests was assessed by one-tailed Spearman's rank correlations. Accordingly, analyzed parameters for choice impulsivity were mean AUC, *k* and A values of individuals, and aggression measures included mean durations of offensive aggression, social investigation, non-social behavior and grooming, as well as number of attacks and attack latency.

2.3.5.4. Test-order effects

For the analysis of possible test-order effects on choice impulsivity, large-reward data from each impulsivity group were analyzed separately using a mixed-model ANOVA with session and delay as within-subjects factors and test order as between-subjects factor.

Potential test-order effects on aggressive behavior were analyzed by non-parametric Mann-Whitney U tests with test-order group as between-subjects factor to compare mean values for duration of offensive aggression, number of attacks and attack latency. Additionally, correlation analyses for

aggression measures were compared for each test-order group using one-tailed Spearman's rank correlations, as performed for the whole group of rats.
Furthermore, impulsivity and aggression measures were analyzed by one-tailed Spearman's rank correlations for the detection of possible test-order effects on the relationship of both behaviors.

2.4. Results

2.4.1. Impulsive choice behavior

A DD paradigm was used to measure impulsive choice behavior in male DA rats. A stable performance in the final five sessions was found in 26 of 32 rats as analyzed by individual repeated measures ANOVAs with session as within-subjects factor (All $F \leq 3.29$, $p > 0.07$, $\varepsilon = 0.27\text{-}0.53$). The remaining six rats were excluded from further analyses, because they did not meet the criterion for stable choice behavior (All $F \geq 3.23$, $p < 0.05$, $\varepsilon = 0.63$).
All rats included into analysis established a preference (> 80 %) for the large reward already during baseline training. An average preference of 96 % ± 5 % SD was determined in the last three training sessions. This preference was maintained throughout all DD sessions (Fig. 2.2) with immediate delivery of the large reward (0-s delay) except for rats of the H-Imp group; their preference decreased on average to 77 %. In this regard, a Kruskal-Wallis test revealed a significant main effect of impulsivity group at the 0-s delay ($H_2 = 6.32$, $p < 0.05$). Further multiple comparisons showed that a significant difference was present between H-Imp and L-Imp rats (Bonferroni corrected, $p < 0.05$), whereas all remaining group comparisons were not significant (Bonferroni corrected, all $p = 1.0$).
In all rats tested, the percent of omissions was less than 20 during the last five DD sessions. Therefore, rats rather chose the small reward over the large reward instead of refraining from responding.
For classification of impulsive behavior, the AUC parameter was used. The average of AUC values for DA rats was 0.54 ± 0.34 SD and the range extended from 0.08 to 0.98 (Fig. 2.3A), i.e., a very high to a very low level of impulsivity, respectively. The majority of rats displayed either L-Imp (n = 12) or H-Imp (n = 11) choice behavior, while only three rats were classified as M-Imp. Mean AUC values were 0.87 ± 0.11 SD for L-Imp, 0.52 ± 0.10 SD (n = 3) for M-Imp and 0.19 ± 0.11 SD for H-Imp rats.
When DD curves of grouped data (large-reward choices) are considered (Fig. 2.3B), a mixed-model ANOVA with session and delay as within-subjects factors and impulsivity group as between-subjects factor revealed a main effect of group ($F_{2,23} = 148.81$, $p < 0.001$), a main effect of delay ($F_{2.59,59.48} = 92.30$, $p < 0.001$, $\varepsilon = 0.65$) and a significant group by delay interaction ($F_{5.17,59.48} = 10.733$, $p < 0.001$, $\varepsilon = 0.65$). Further, multiple comparisons revealed highly significant differences between the three impulsivity groups (Bonferroni corrected, all $p < 0.001$) and between the different delays (Bonferroni corrected, all $p < 0.01$). Thus, with increasing delay to the large reward, the degree of discounting differed significantly across all three impulsivity groups (Fig. 2.3). H-Imp rats displayed the highest delay aversion and devalued the large reward already at lower delay times compared to M-Imp or L-Imp rats. The latter group thereby shows the highest preference for the large reward with increasing delay. No significant main effect was observed for session ($F_{2.54,58.41} = 0.24$, $p = 0.84$, $\varepsilon = 0.64$) or all remaining interactions (All $F < 1.6$, $p > 0.19$, $\varepsilon = 0.50\text{-}0.64$).
In summary, L-Imp rats show a high tolerance towards increasing delays and maintain this tolerance over a long period of time compared to M-Imp or H-Imp individuals of the DA strain (Fig. 2.2, Fig 2.3).
However, the calculation of the AUC parameter does not take into account the shape of the discounting curve. Therefore, we additionally analyzed preference data by fitting a hyperbolic

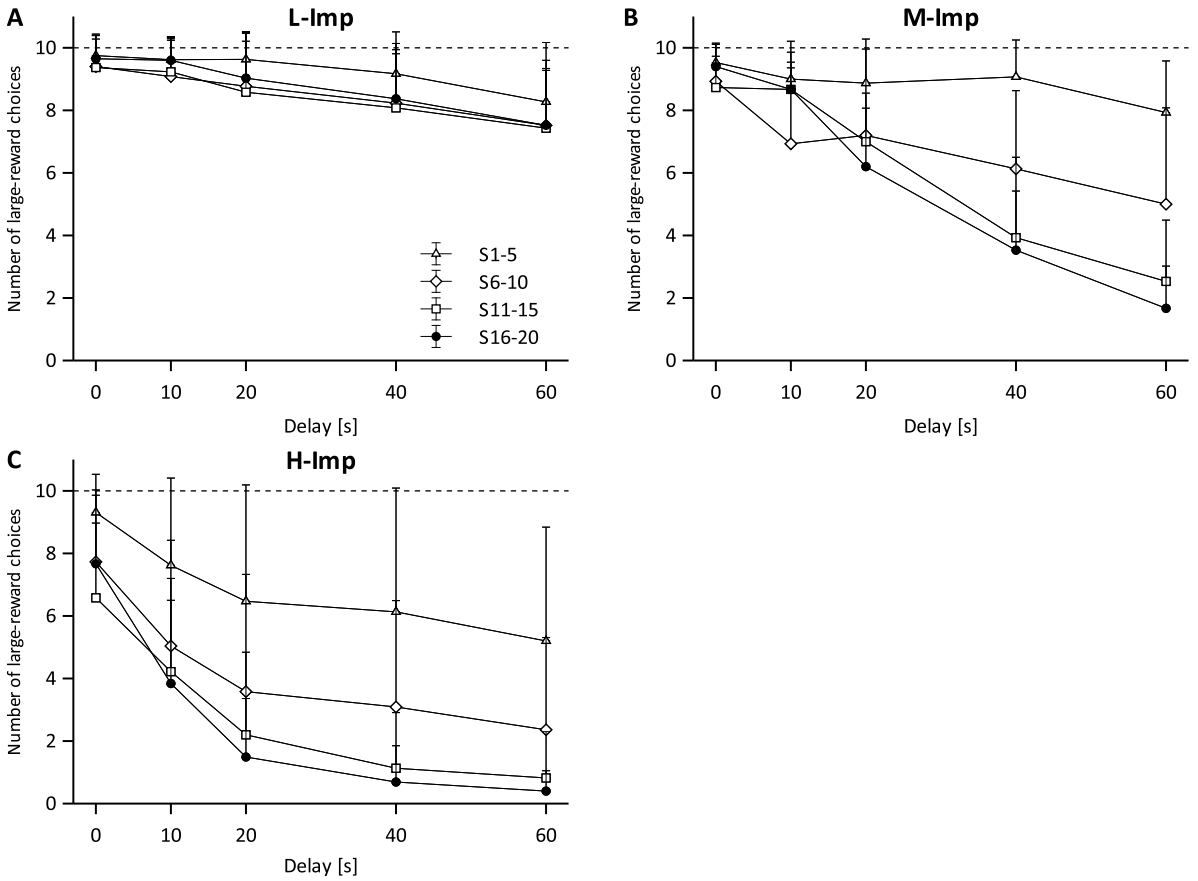

Figure 2.2 | **Impulsive choice behavior in male Dark Agouti rats during the course of the delay-discounting (DD) task**. DD curves illustrate choices for the large reward as a function of delay. Large-reward choice data for low-impulsive (**A**, L-Imp; n = 12), medium-impulsive (**B**, M-Imp; n = 3) and highly-impulsive (**C**, H-Imp; n = 11) groups are shown in five-session intervals. L-Imp rats maintained a strong preference for the large reward from the first to the last sessions, whereas M-Imp and H-Imp rats decreased their preference steadily throughout the DD task and established stable behavior in the last DD sessions. H-Imp rats additionally decreased their preference at the 0-s delay, and generally displayed the greatest decrease in large-reward choices during the course of the DD task. Data are expressed as mean + SD. S, Session.

discounting function to it. From this, we derived estimates for the sensitivity of reinforcer delay (*k* value) and reinforcer amount (A value). The average of *k* values for all DA rats was 0.21 ± 0.74 SD and the individual range extended from 0.00 to 7.82. Individual estimates of A values ranged from 5.68 to 9.95 with an overall mean value of 8.92 ± 1.74 SD.

AUC, *k* and A values were all significantly intercorrelated (Spearman's correlation, one-tailed; Table 2.1), illustrating a good concordance between impulsivity measures. The AUC and *k* parameters were negatively correlated, because both are inversely proportional to each other. Higher AUC values reflect a low level of impulsivity while *k* values decrease with decreasing choice behavior. Accordingly, the A parameter correlates positively with AUC and negatively with *k* values, such that increased choice impulsivity is associated with decreased sensitivity to reinforcer magnitude. When sensitivity to delay, reflected by the *k* value, remains constant, while the A value increases, the preference curve is shifted upwards and thus a lower impulsivity level is observed. When the A value remains constant, a flattening of the discounting curve (higher *k* value) results in a lower impulsivity level. Thus, parameters *k* and A can affect impulsive behavior independently (also see: Ho et al., 1999; Pitts and Febbo, 2004).

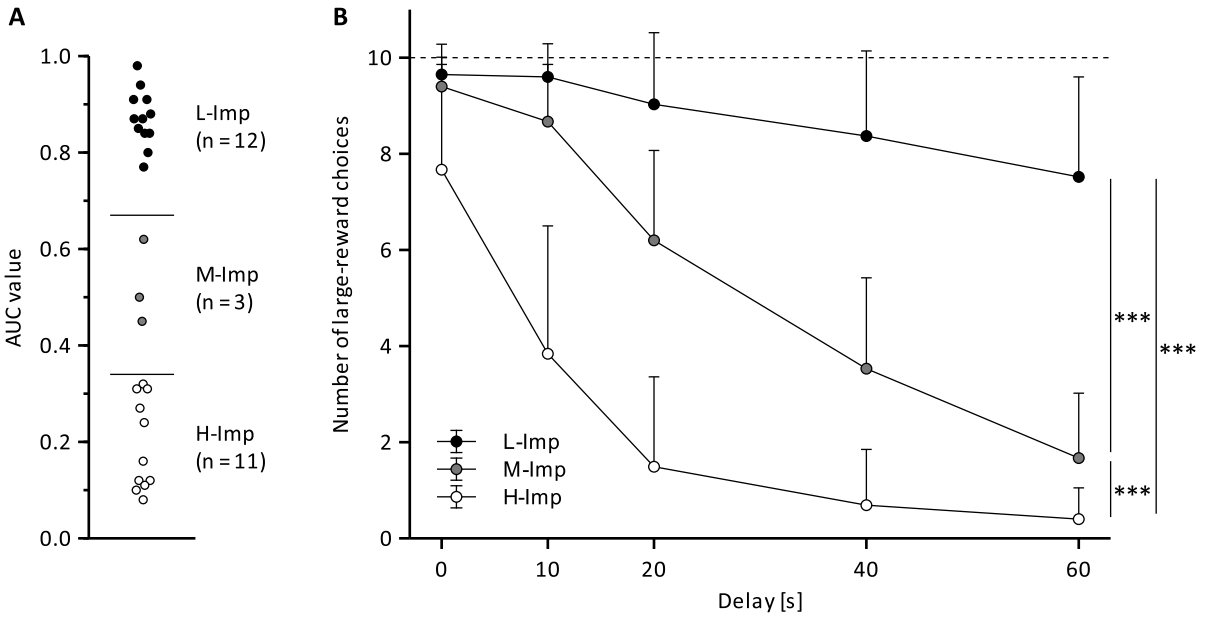

Figure 2.3 | **Impulsive choice behavior in male Dark Agouti rats. A**, Mean area under curve (AUC) values of individuals (n = 26) are shown measured by a delay-discounting (DD) paradigm (last 5 out of 20 sessions). Horizontal lines represent the borders between impulsivity groups: Low (L-Imp), medium (M-Imp) or highly impulsive (H-Imp). **B**, DD curves illustrate choices for the large reward as a function of delay in the DD task (last 5 out of 20 sessions). Data for discounting curves are grouped by impulsivity level. Data are expressed as mean + SD. ***p < 0.001.

2.4.2. Inter-male territorial aggression

Rats performed four RI tests and mean data from all tests was used for analysis. In total, residents spent half of the time in non-social behaviors (53.9 % ± 20.0 % SD), followed by social investigations (30.3 % ± 12.0 %), offensive-aggressive interactions (12.7 % ± 15.9 %) and grooming (3.1 % ± 4.4 %). Although the percentage of offensive aggression is relatively low, an inter-individual range of 0-41.1 % (SD range 0-16.3 %) was observed. Thereby, the majority of rats (n = 17) spent up to 10 % of time in offensive-aggressive behavior, while the remaining rats (n = 9) were engaged in aggressive behavior from 20 % to 45 % of time (Fig. 2.4). With reference to aggression score classes defined by de Boer and colleagues (2003), the larger group represents L-Agg individuals and the smaller group represents M-Agg individuals. Aggression levels above 55 %, corresponding to a H-Agg phenotype, were not observed.

L-Agg and M-Agg groups were subsequently analyzed for differences within the four main behavioral categories from RI tests (Fig. 2.5). Mann-Whitney U tests revealed significantly higher times spent in aggressive behavior for M-Agg rats compared to L-Agg rats (Mdn: L-Agg = 1.43, M-Agg = 31.68, U = 0, $p < 0.001$, r = 0.81) as well as significantly lower times spent in non-social behaviors (Mdn: L-Agg = 64.03, M-Agg = 37.31, U = 8, $p < 0.001$, r = 0.72). Social investigations and grooming did not differ significantly between the two groups of rats (social: Mdn: L-Agg = 26.75, M-Agg = 30.66, U = 63, p = 0.491, r = 0.14; groom: Mdn: L-Agg =2.77, M-Agg = 1.54, U = 46, p = 0.107, r = 0.32). The number of attacks was significantly increased in M-Agg compared to L-Agg rats (Mdn: L-Agg = 0.25, M-Agg = 4.25, U = 5, $p < 0.001$, r = 0.77) and this was accompanied by a significant decrease in attack latency for the L-Agg group (Mdn: L-Agg = 551, M-Agg = 164, U = 0, $p < 0.001$, r = 0.82). Here, the number of attacks for all rats ranged from 0 to 11. L-Agg rats attacked on average 0.63 times, while

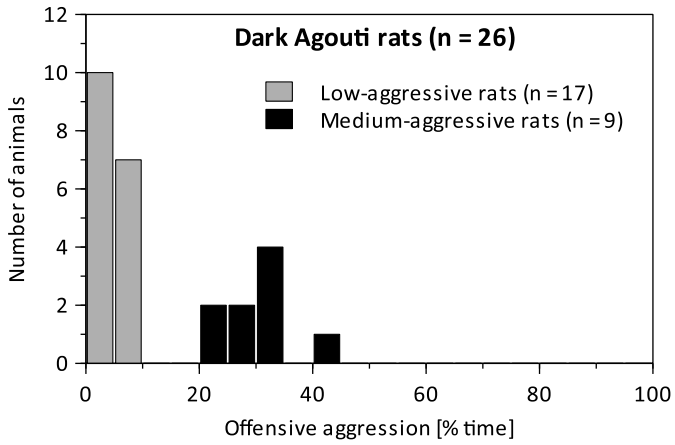

Figure 2.4 | **Frequency distribution of offensive aggressive behavior of male Dark Agouti rats.** Data are mean values of individuals from four resident-intruder tests. Columns represent 5 % bins. A low- (L-Agg) and a medium-aggressive (M-Agg) group were determined for rats of the present study according to aggression score classes defined by de Boer et al. (2003).

the mean attack number for M-Agg rats was approximately six times higher at 3.75. Mean attack latencies were 537 s in L-Agg and 174 s in M-Agg rats with a range of 116 s to 600 s and 24 s to 600 s, respectively. Hereby, 600 s correspond to the end of a RI test, which means that the rat did not perform any aggressive act.

Additionally, statistical analyses were performed to determine the interrelationship of aggression parameters independent of aggression score classes. Correlation analysis of the three main aggression measures, i.e., duration of offensive aggression, attack number and attack latency, revealed highly significant correlations between all parameters. The time spent in aggressive interactions was positively correlated with number of attacks (Spearman's correlation, one-tailed; $r = 0.900$, $p < 0.01$, $n = 26$). Highly significant, negative correlations were found between attack latency and number of

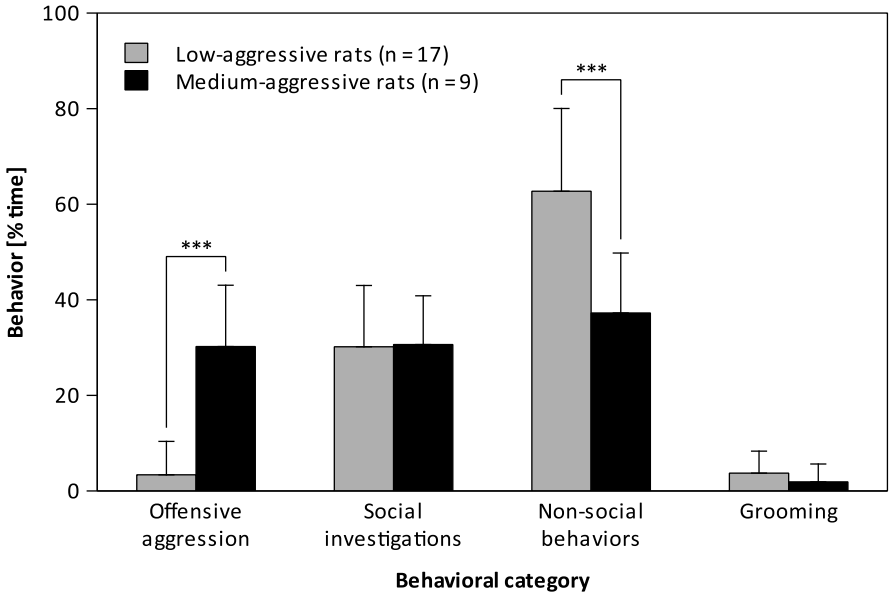

Figure 2.5 | **Behavioral measures during resident-intruder tests in male Dark Agouti rats.** Low- (L-Agg) and medium-aggressive (M-Agg) rats differ significantly in the time spent in aggressive behavior as well as in non-social behaviors. Data of all four RI tests are pooled and expressed as mean + SD. ***$p < 0.001$.

attacks as well as duration of offensive aggression (Spearman's correlation, one-tailed; Attack number: r = -0.925, p < 0.01; Offensive aggression: r = -0.948, p < 0.01; n = 26). Accordingly, shorter attack latencies resulted in an increased number of attacks and both of these measures coincide with more time spent in offensive aggression. Thus, resident rats that displayed a short attack latency also attacked more frequently and engaged in aggressive behaviors for a longer period of time during confrontation with an intruder rat.

2.4.3. Relationship between aggression and impulsive choice

To evaluate the relationship between impulsive choice behavior and offensive aggression as well as other measures from territorial confrontations, a correlation analysis was performed. For the whole group of rats (n = 26), no significant correlations were found between AUC or *k* values and the four main behavioral categories of RI tests (Table 2.1). Moreover, neither attack latency nor attack number were correlated with AUC or *k* values (Table 2.1). In this regard, our results show an absence of correlation between aggression and impulsivity.

A parameter estimates, however, were positively correlated with number of attacks in RI tests (Table 2.1), such that the higher the number of attacks, the higher the A value, i.e., the preference for the large reward when there is no delay to the large reward. Hence, attack number is related to sensitivity to reinforcer magnitude, whereas no relation was detected regarding sensitivity to delay (*k* value). Concerning the other aggression parameters duration of offensive aggression and attack latency, correlations with the A value were not significant but showed a positive and a negative trend, respectively (Table 2.1). These results indicate that an increased preference for the large reward may coincide with increased duration of offensive aggression and decreased attack latency which again indicates an earlier onset of aggressive behavior.

Table 2.1 | Spearman's rank correlation coefficients for behavioral measures of impulsivity and measures from resident-intruder tests in male Dark Agouti rats (n = 26).

	Delay discounting		**Resident-intruder test**					
	k	A	Attack number	Attack latency	Offensive aggression	Social invest.	Non-social behaviors	Grooming
AUC	**-0.971****	**0.715****	0.169	-0.152	0.191	0.091	-0.173	0.151
k		**-0.594****	-0.173	0.153	-0.199	-0.134	0.221	-0.141
A			**0.381***	-0.260$^{\#}$	0.268$^{\#}$	0.185	-0.249	0.132

Note. A, estimate of sensitivity to reinforcer magnitude at 0-s delay (y-intercept); AUC, area under curve; *k*, estimate of sensitivity to delay (steepness of discounting curve); Social invest., Social investigations. *p < 0.05, **p < 0.01, $^{\#}$p < 0.1 (trend), one-tailed. Significant correlations are indicated in bold.

Visual inspection of the level of impulsivity represented by AUC values and the duration of offensive aggression reflects a widespread distribution for all individuals rather than a linear relationship between both behaviors (Fig. 2.6), which is in accordance with the statistical results. Regarding behavioral classes for impulsivity and aggression (Fig. 2.6, dashed lines on axes), six phenotypic combinations of impulsive choice behavior and offensive aggression were observed within the detected ranges of impulsivity from low to high levels and aggression reaching from low to medium levels.

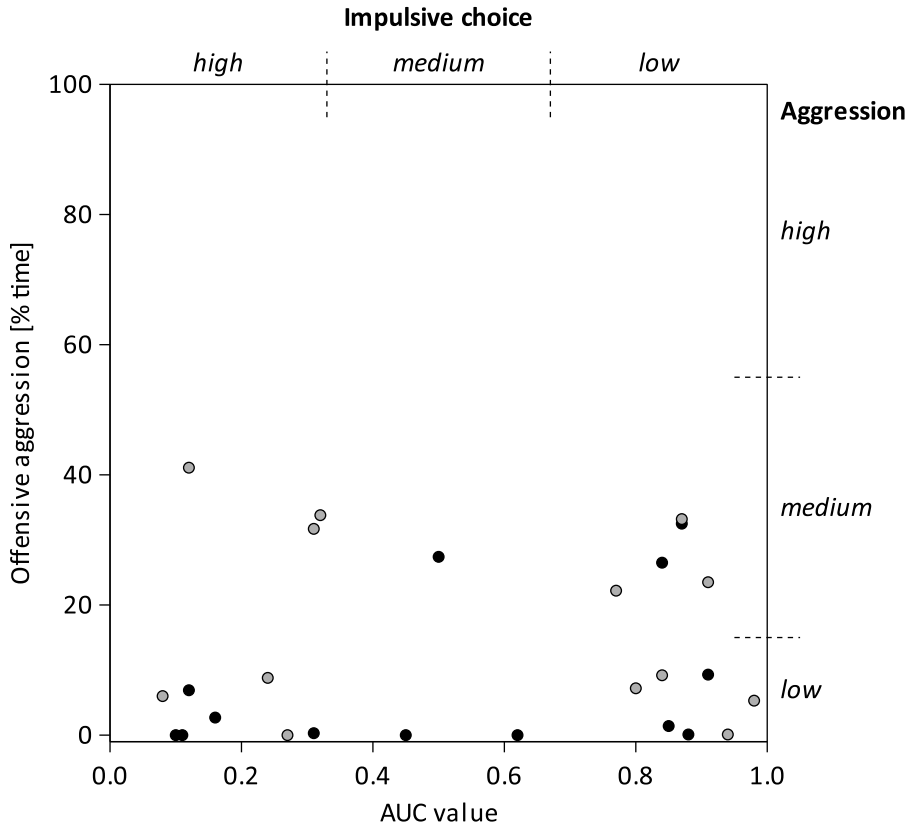

Figure 2.6 | **Distribution of impulsive and aggressive phenotypes in male Dark Agouti rats.** Plotted data represent mean area under curve (AUC) values on the horizontal axis and average duration of offensive aggression on the vertical axis for individuals (n = 26). Untransformed data is shown for both parameters. Dashed lines on the upper horizontal and right vertical axes correspond to borders of behavioral categories for choice impulsivity and aggression, respectively. Increasing AUC values correspond to decreasing levels of impulsivity. Data points are color coded for indication of test-order conditions: gray circles, DD-RI group (n = 13); black circles, RI-DD group (n = 13). DD, Delay-discounting task; RI, Resident-intruder tests.

2.4.4. Test-order effects

2.4.4.1. Test-order effects on impulsivity

For the assessment of test-order effects on impulsive choice behavior, data on large-reward choices were analyzed by mixed-model ANOVAs for each impulsivity group with session and delay as within-subjects factors and test order as between-subjects factor. Tests on within-subjects factors revealed a main effect of delay for each of the three impulsivity groups of rats (L-Imp: $F_{4,40} = 20.97$, $p < 0.001$, DD-RI: n = 7, RI-DD: n = 5; M-Imp: $F_{1.63,3.25} = 47.04$, $p < 0.01$, $\varepsilon = 0.41$; H-Imp: $F_{4,36} = 81.18$, $p < 0.001$, DD-RI: n = 6, RI-DD: n = 5). No main effect of session (All $F < 1.00$, all $p > 0.42$; M-Imp: $\varepsilon = 0.41$) or test order by delay or by session interactions were observed in either group (All $F < 1.72$, all $p > 0.16$; $\varepsilon = 0.11$-0.37). For the between-subjects factor test order, no significant differences were found for L-Imp or H-Imp groups of rats (L-Imp: $F_{1,10} = 0.39$, $p = 0.548$; H-Imp: $F_{1,9} = 1.17$, $p = 0.307$). Test-order effects for the M-Imp group could not be determined statistically, because only one test-order group was present (DD-RI: n = 0, RI-DD: n = 3).

Hence, there were no statistically significant effects on the preference behavior of Dark Agouti rats between both test-order groups, but the lack of M-Imp individuals in the DD-RI test order group might

suggest that rats that performed the DD paradigm without an experience in inter-male confrontations display a clear preference for either the small or the large reward.

2.4.4.2. Test-order effects on aggression

Statistical analysis of aggression measures by Mann-Whitney U tests revealed no significant effects of test order for all three measures (attack number: Mdn: DD-RI = 1.50, RI-DD = 0.25, U = 64, p = 0.311, r = 0.21; attack latency: Mdn: DD-RI = 439.68, RI-DD = 536.82, U = 57, p = 0.169, r = 0.28), although the duration of offensive aggression showed a non-significant trend towards a longer duration of aggressive behavior in the DD-RI group (Mdn: DD-RI = 9.20, RI-DD = 1.43, U = 48.5, p = 0.064, r = 0.36). Additional correlation analysis of aggressive behavior for each test-order group revealed highly significant results between all parameters, similar to present results for the whole group of rats (see section 2.4.2.). In all cases, duration of offensive attacks correlated positively with attack number, while attack latency correlated negatively with the other two parameters (Table 2.2). Hence, the shorter the attack latency, the more time is spent in offensive aggressive behavior and the more attacks are performed by the resident rat. Overall, present results indicate that aggression measures are not affected by test-order condition.

Table 2.2 | **Spearman's rank correlation coefficients for aggression measures in male Dark Agouti rats of both test-order groups.**

	Behavioral measure	Offensive aggression	Attack number	Attack latency
DD-RI n = 13	Offensive aggression	1	**0.828****	**-0.916****
	Attack number		1	**-0.893****
	Attack latency			1
RI-DD n = 13	Offensive aggression	1	**0.949****	**-0.950****
	Attack number		1	**-0.983****
	Attack latency			1

Note. DD, Delay-discounting task, RI, Resident-intruder tests. **p < 0.01, one-tailed. Significant correlations are indicated in bold.

2.4.4.3. Test-order effects on relations between both behaviors

When DD performance preceded aggression tests (DD-RI), no significant correlations between impulsivity and aggression parameters were examined (Table 2.3). For the RI-DD group, however, several parameters showed significant correlations (Table 2.3). In particular, duration of offensive aggression correlated positively with AUC values and negatively with *k* values, i.e., individuals with increased choice impulsivity spent less time in offensive aggression. Additionally, a trend was observed towards a negative correlation between *k* values and number of attacks. With regard to the other categories from RI tests, durations of social investigations and non-social behaviors both correlated with AUC and *k* values, such that increased choice impulsivity (decrease in AUC and increase in *k*) coincides with shorter durations of social investigations and with longer durations of non-social behaviors.

For the A parameter, a positive correlation was found with attack number and a negative correlation with non-social behaviors in RI tests. Thus, the higher the preference for the large reward when no

delay is imposed, the higher the number of attacks and the less time was spent on non-social behaviors. Additionally, trends towards positive correlations between A and both offensive aggression and social investigations were found, as well as a trend towards a negative correlation with attack latency.
Taken together, test-order effects were only observed for the group of rats that performed RI tests prior to the DD paradigm, but not for rats with the reversed test order.

Table 2.3 | Spearman's rank correlation coefficients for behavioral measures of impulsivity and aggression in male Dark Agouti rats of both test-order groups.

		Delay discounting		Resident-intruder test					
		k	A	Attack number	Attack latency	Offensive aggression	Social invest.	Non-social behaviors	Grooming
DD-RI n = 13	AUC	**-0.978*** **	**0.670** **	-0.158	-0.011	-0.176	-0.341	0.192	0.264
	k		**-0.571***	0.188	-0.006	0.170	0.236	-0.104	-0.319
	A			0.119	-0.083	-0.049	0.005	0.077	0.077
RI-DD n = 13	AUC	**-0.967*** **	**0.676** **	0.376	-0.329	**0.486***	**0.571***	**-0.637** **	0.055
	k		**-0.566***	-0.382$^{\#}$	0.361	**-0.503***	**-0.588***	**0.709** **	0.005
	A			**0.480***	-0.393$^{\#}$	0.074$^{\#}$	0.451$^{\#}$	**-0.588***	0.214

Note. A, estimate of reinforcer amount at 0-s delay (y-intercept); AUC, area under curve; *k*, estimate of sensitivity to delay (steepness of discounting curve); Social invest., Social investigations; DD, Delay discounting; RI, Resident-intruder tests. *p < 0.05, **p < 0.01, ***p < 0.001, $^{\#}$p < 0.1 (trend), one-tailed. Significant correlations are indicated in bold.

Additional visual inspection on the distribution of impulsive and aggressive phenotypes with regard to both test-order conditions has shown that five out of six phenotypes that occurred for the whole group of rats were distinguished in the RI-DD group (Fig. 2.6, black circles). In particular, a H-Imp/M-Agg phenotype was not present in this test-order group. In the DD-RI group (Fig. 2.6, gray circles), the following four behavioral combinations were present: L-Imp/L-Agg, L-Imp/M-Agg, H-Imp/L-Agg and H-Imp/M-Agg. Due to the underrepresentation and the uneven distribution of M-Imp rats, data points in the DD-RI group do not follow a monotonic distribution but rather split into two groups of data along the impulsivity axis.

2.5. Discussion

2.5.1. Impulsive choice behavior

Within the DA rat strain, the distribution of individual AUC values revealed that impulsive choice behavior extended from very low to very high levels. Thereby, the majority of rats displayed choice behavior in either the L-Imp or the H-Imp scale, whereas only a few individuals were classified into the M-Imp category. This demonstrates a high range of inter-individual differences in choice impulsivity which mainly manifests at the extreme ends of the behavioral spectrum. Such a dichotomy could be important for the analysis of neural correlates of impulsive behavior.

Evaluation of choice impulsivity using a DD procedure generally requires a high preference for the reinforcer in order to assess tolerance towards delays. In the current study, rats established a high preference for the large reward during baseline sessions and maintained this preference during DD with immediate delivery of the large reward. Some H-Imp individuals, however, decreased this preference as DD sessions continued. One possible explanation for this effect could be the design of the DD task, because in four of five session blocks a reinforcer delay was included. This predominant delay presentation, regardless of delay length, could have caused a shift towards the immediate reward in rats that display high delay aversion. Additionally, sessions always ended with the highest possible delay, which potentially caused H-Imp rats to expect a delay at the beginning of a following session. Such between-session carry-over effects were also reported in previous studies (Fox et al., 2008; Madden et al., 2008). An alternative explanation for the effects on large-reward preference may be related to behavioral inflexibility (Schwager et al., 2014). Nevertheless, rats performed a minimum of omissions in DD sessions, which implies that H-Imp rats switched preferences from the large to the small reward instead of choosing none of these options. Thus, differences in impulsivity in DA rats are presumably not driven by motivational factors, such as the interest in an appetitive reinforcer or an overall motivation for lever pressing.

The DA rat strain was often used for immunological experiments due to its susceptibility to autoimmune diseases (Foster et al., 2009; Merrill et al., 2009; Muschter et al., 2015; Eriksson et al., 2016), whereas experiments on behavior are less often found. One study by Saadat and colleagues (2006) found that MDMA increased impulsive action on a short-term basis, but impulsive choice or individual differences in impulsive action were not examined.

Another recent study by Alonso et al. (2020) examined choice impulsivity in DA rats and found a high inter-individual range as well as a high preference for the large reward at the 0-s delay consistent with present findings. This agreement of results is observed although procedural differences exist between both studies. Compared to the current study, reinforcer amount differed between one and five pellets, the maximum delay was shorter (40 s) and rats had to perform nose pokes instead of lever presses.

In the present study, L-Imp rats even tolerated waiting times up to one minute repeatedly to receive a large reward and maintained this preference over the entire course of the DD task. For comparison, H-Imp and M-Imp rats markedly reduced their large-reward preference and established a stable performance only in the last DD sessions. Due to this high tolerance towards delayed reinforcers in several individuals of the DA strain and the high inter-individual range, DA rats, so far, hold a special position among inbred strains.

Previous studies in inbred rats mainly reported a single class of impulsivity for a strain rather than differing degrees for individuals (Anderson and Woolverton, 2005; Wilhelm and Mitchell, 2009; Huskinson et al., 2012; Stein et al., 2012; Richards et al., 2013). These studies often focused on Lewis and Fischer 344 rats either revealing increased choice impulsivity of Lewis rats (Anderson and Woolverton, 2005; Huskinson et al., 2012; Stein et al., 2012) or reporting no differences between both strains (Madden et al., 2008; Wilhelm and Mitchell, 2009; Richards et al., 2013).

Individual differences in outbred rat strains were previously reported for Lister hooded (Robinson et al., 2009), Long Evans (Simon et al., 2013; Zeeb et al., 2016; Moschak and Carelli, 2017), Sprague-Dawley (Dellu-Hagedorn, 2006) and Wistar Han rats (Diergaarde et al., 2008; Dellu-Hagedorn et al., 2018; Ucha et al., 2019; Alonso et al., 2020). Individuals of these aforementioned strains were often classified into distinct impulsivity groups due to a wide range of choice behavior. Especially, Dellu-Hagedorn and colleagues (2018) found a high inter-individual variability in Wistar rats with on average 6 % to 99 % of large-reward choices. These diverse manifestations of choice impulsivity in outbred rats together with impulsivity group classifications were similar to the current results in DA rats. Thus, the

DA strain appears to have maintained a high variability in impulsive choice behavior as usually found in outbred strains.

2.5.2. Inter-male territorial aggression

In the RI test, male DA rats constituted two groups, one with a medium level of aggression towards an unfamiliar male conspecific and the other one with a low level or no aggressive behavior at all. Highly-aggressive individuals were not observed throughout the experiment. The expected behavioral range of aggressive behavior, as previously found in our laboratory (Radant, 2010), was therefore not confirmed for the examined population of animals. Furthermore, Radant (2010) found a different distribution of individuals within aggression score classes, i.e., the majority of DA rats were classified as M-Agg, whereas in the present study, 65 % of rats displayed L-Agg behavior. This shift towards less aggression may reflect natural variations between generations of DA rats bred in our laboratory (also see de Boer et al., 2017).
The absence of H-Agg phenotypes in laboratory rats was likewise reported for the Wistar Han outbred strain, while the whole aggressive spectrum was identified in wild-type Groningen (WTG) rats (de Boer et al., 2003) and Long-Evans outbred rats (Blanchard et al., 1988). Hence, a narrow aggressive profile as found in DA (present study) or Wistar rats (de Boer et al., 2003), may as well be due to domestication (Koolhaas, 2010), independent of in- or outbred conditions, and may lead to a reduction of genetic and phenotypic variability (Visscher et al., 2008).
The L-Agg and M-Agg groups, identified in the current study, did not only differ by duration of offensive aggression but also by duration of non-social behaviors such as cage exploration. The increased duration of offensive aggression in M-Agg rats seems to be compensated in L-Agg rats by non-social behaviors. In this aspect, behavioral profiles of DA rats differ from profiles reported for WTG and Wistar rats (de Boer et al., 2003). In the two latter strains, both L-Agg and M-Agg rats decreased the time of social investigations when durations of aggressive behaviors increased, while non-social behaviors did not differ (de Boer et al., 2003). In contrast, in the present study, the duration of social investigations did not differ between aggression groups, and thus DA rats explored their opponents for an equal amount of time without engaging in aggressive acts. Taken together, the DA inbred strain shows a limited range of aggressive individuals compared to a previous population of rats from our laboratory and a different behavioral profile compared to outbred or wild-type strains.

2.5.3. Relationship between impulsivity and aggression

In male DA rats, several phenotypic combinations of impulsive choice and aggression were present, while an overall correlation between these two traits was not observed. Our results are therefore in contrast to the general hypothesis that increased aggression is associated with increased impulsivity (van den Bergh et al., 2006b; Cervantes and Delville, 2007).
Considering different facets of impulsivity, positive or negative correlations as well as no correlations were reported for impulsive-aggressive phenotypes (Flagel et al., 2010; Kerman et al., 2011; Coppens et al., 2014). For example, in rats selectively bred for the response in a novel environment, high-responder rats displayed enhanced aggressive behavior in confrontations with an intruder (Kerman et al., 2011) along with enhanced impulsive action in a DRL task, while delay aversion measured in a DD task was decreased (Flagel et al., 2010). Thus, the authors have shown that aggression was positively associated with impulsive action and negatively with impulsive choice, which may be a result of

selection of impulsive-aggressive phenotypes during breeding in this rat line. However, DA rats of the present study were not selectively bred for a certain behavior and have shown a naturally occurring spectrum of aggression with fluctuations between generations. While choice impulsivity and aggression were generally uncorrelated in DA rats, a potential relationship between aggression and impulsive action remains to be investigated. Furthermore, a potential correlation between aggression and impulsive choice in DA rats may remain undetected due to the absence of H-Agg individuals. The study by Coppens et al. (2014), for instance, found no relation between aggression and response inhibition in a DRL task in WTG rats that displayed a normal range of aggression. Violent individuals of the latter strain, however, showed an increased intolerance towards delays in the DRL task (Coppens et al., 2014). Hence, violent or H-Agg individuals may be more prone to delay aversion. While a wide range of choice impulsivity seems to be preserved in DA rats, a shift towards less aggressive behavior was observed in the examined DA population compared to earlier work from our laboratory (Radant, 2010). The lack of association between territorial aggression and choice impulsivity seen in this strain might therefore be a consequence of varying individual ranges of both traits.
Investigations in Wistar rats (van den Bergh et al., 2006b) and golden hamsters (Cervantes and Delville, 2007) were shown to be in agreement with the general hypothesis that both traits change concurrently in the same direction. However, the latter study only included individuals into analysis that exhibited aggressive acts, leaving out hamsters that performed no attacks. It is unclear whether this relationship applies for non-aggressive individuals as well. In DA rats, half of the animals that performed no attacks still engaged in other aggressive acts like offensive upright (data not shown). Moreover, we found a relation between number of attacks from aggression tests and the sensitivity to reinforcer amount reflected by the A parameter estimate of the hyperbolic discounting function fitted to the large-reward data from the DD task. With increasing number of attacks, A values increased as well, i.e., individuals that attacked more often also preferred the large reward at the no delay condition. Thus, aggression in DA rats was positively correlated with the sensitivity to reward magnitude, a specific estimate which is inversely proportional to the level of choice impulsivity. Furthermore, trends to decreased duration of offense and to increased attack latency were observed with increasing A value. These results in DA rats illustrate independent contributions of reinforcer delay and magnitude to choice impulsivity, which were also discussed in previous publications (Ho et al., 1999; Pitts and Febbo, 2004).

2.5.4. Test-order effects on choice impulsivity and territorial aggression

When impulsivity and aggression measures are considered separately, no statistically significant test-order effects were observed in the present study. For the M-Imp group, however, statistical analysis could not be performed because in the DD-RI group no individual classified as medium impulsive. Hence, it might be suggested that rats that performed the DD paradigm without an experience in inter-male confrontations display a clearer preference for either the small or the large reward when a delay is imposed, but it is also possible that this effect is only coincidental due to the relatively small sample sizes within both test-order groups.
With regard to relations between both behaviors, significant test-order effects were observed for the RI-DD, but not for the DD-RI group. In the former, the duration of offensive aggression correlated with both impulsivity parameters AUC and *k*. Hence, in these rats, decreased levels of aggression were associated with an increase in choice impulsivity and vice versa. This is in contrast to the general hypothesis that increased aggression is associated with increased impulsivity (van den Bergh et al., 2006b; Cervantes and Delville, 2007), but similar results were reported for selectively bred high responder rats (also see section 2.5.3.) (Flagel et al., 2010; Kerman et al., 2011).

One aspect that may account for test-order effects are winning or losing experiences of a resident in an established territory. Repeated winning in quick succession was previously shown to progressively enhance aggressive behavior potentially due to a rewarding effect of aggression (Hsu et al., 2006; de Boer et al., 2009; Kerman et al., 2011; Falkner et al., 2016).
In the RI-DD group of the present study, offensive behavior expressed during aggression testing may have had such a rewarding effect which may have influenced operant behavior tested subsequently in the DD task. Moreover, present investigations have shown that in the RI-DD group the number of attacks was positively correlated with the A parameter from hyperbolic curve fitting and thus with the sensitivity to reinforcer magnitude. Hence, rats that attacked more frequently in RI confrontations, showed a strong preference for the large reward when no delay was present. Such a relation was also observed for the whole group of rats (see section 2.5.3.), but not for rats of the DD-RI group.
Collectively, DA rats with an experience in inter-male confrontations and an increased level of aggression also displayed a generally high preference for the large reward in the DD task when no delay was present accompanied by a high tolerance towards delays. Previous experiences from RI tests thus appear to play a role in the behavioral outcome of a subsequent DD task with regard to both reinforcer delay and magnitude.
However, test-order effects may not exclusively depend on the experience in aggressive behaviors, but may be associated with non-aggressive behaviors from inter-male confrontations as well. Within the RI-DD group, all three impulsivity measures were correlated with non-social behaviors and AUC and *k* values were correlated with social investigations. Accordingly, individuals with increasing levels of impulsivity spent less time in social investigations and engaged in non-social behaviors instead. Hence, DA rats with an enhanced intolerance towards delays in the DD task also tended to avoid social contacts during inter-male confrontations. As a result, each of these behavioral aspects might serve the avoidance of unpleasant or frustrating situations.

2.5.5. Conclusion

Taken together, results from the present study and previous investigations demonstrate the diversity of impulsive-aggressive manifestations in rodents with respect to different aspects of impulsivity as well as its dependency on breeding such as the choice of an in- or outbred strain or selective breeding of a specific trait.
Male DA rats showed stable choice impulsivity together with a high inter-individual range for this trait resulting in high-, medium- and low-impulsive rats. Classification of offensive aggression revealed two groups with either low or medium aggression levels; highly-aggressive rats were not present. Thus, DA rats seem to have maintained a high variability in choice impulsivity as usually found in outbred strains, while aggressive behavior does not show the full spectrum, and the occurrence of highly-aggressive individuals appears to vary between generations. The absence of an overall correlation of both behaviors may be subjected to these variations in behavioral ranges. The broad behavioral spectrum of impulsive choice in rats of the DA strain as well as its clear distinction between low and high extremes may be an advantage in future investigations on the neuronal correlates of impulsivity.
However, territorial aggression and choice impulsivity were linked in DA rats in a parameter-specific manner and were partly dependent on test order. On the one hand, in all rats, an enhanced number of attacks was associated with an increased choice for the large reward when delivered immediately, and thus attacks were related to changes in sensitivity to reward magnitude, potentially resulting in changes in impulsive choice behavior. On the other hand, the experience in inter-male confrontations seemed to affect choice impulsivity in terms of reward magnitude sensitivity and delay sensitivity.

Further research in the DA inbred strain is needed to more thoroughly determine the influence of reward- and delay-related measures associated with impulsive choice. Additionally, DA rats may be useful for future investigations on the interplay of impulsivity and aggression and on the identification of control circuits and structures in the brain.

Chapter 3

Comparative analysis of c-Fos expression levels in impulsive choice behavior and territorial aggression in male Dark Agouti rats

Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

3.1. Abstract

Several brain regions have been implicated in the regulation of impulsive choice behavior and territorial aggression in rodents, even suggesting overlaps in their neuroanatomical bases. These areas particularly include frontal cortex regions, subcortical structures as well as output structures of monoaminergic neurotransmitters. In the present study, the contribution of distinct brain regions underlying these behaviors were investigated in male Dark Agouti (DA) rats following behavioral performance. In a previous study, we have shown that individuals of this inbred rat strain display natural variations in choice impulsivity as assessed by a delay-discounting (DD) paradigm and inter-male offensive aggression measured in resident-intruder (RI) confrontations. Here, we investigated alterations in neuronal activation patterns using immunohistochemical detection of the c-Fos protein in rats with varying levels of behavior, i.e., low- and highly-impulsive, as well as low- and medium-aggressive. It was found that performance of the DD task and RI tests differentially altered neuronal activation patterns and that an overlapping effect was only observed in the ventral orbital (VO) subregion of the frontal cortex. This area was particularly found to play a role in increased levels of impulsivity, and at the same time in the suppression of aggressive behavior. This finding potentially indicates common regulatory mechanisms within highly-impulsive and low-aggressive phenotypes mediated by the orbitofrontal cortex, whereas the remaining results point towards a different neuroanatomical basis for choice behavior and territorial aggression in this rat strain. Alterations in the number of 5-HT_{1A}-expressing neurons were exclusively found within the ventral subregion of the dorsal raphe nucleus, but these were independent of impulsivity and aggression levels and thus appear to be implicated in general aspects of behavior linked to the performance of both tasks.

3.2. Introduction

Impulsive and aggressive behaviors are common components of the natural behavioral repertoire of healthy individuals. Positive associations between both behaviors have been reported frequently in the literature, especially with maladaptive forms (van den Bergh et al., 2006b; Cervantes and Delville, 2007; Nelson and Trainor, 2007). However, the multifaceted nature of impulsivity as well as aggression make the evaluation of their functional relationship more complex. In our own preceding study, for example, we found that territorial aggression in inter-male confrontations was not significantly related to impulsive choice behavior in male individuals of the Dark Agouti (DA) inbred rat strain (chapter 2 of this dissertation).

In general, the construct of impulsivity comprises a variety of unplanned actions without forethought, often categorized into impulsive action and impulsive choice (Evenden, 1999; Smith et al., 2015). The focus of the present study lies on the latter form which is characterized as the tendency to prefer a smaller, immediate reward over a larger, but delayed reward (Odum, 2011; Robbins and Dalley, 2017).

Impulsive aggression, in contrast to the premeditated and controlled form of instrumental aggression, represents a context-based reaction to immediate threats or challenges, which is also often related to as reactive type of aggression (Cervantes and Delville, 2007; Siever, 2008; Neumann et al., 2010). However, it remains to be fully elucidated which form of impulsivity predominantly contributes to impulsive-aggressive phenotypes in healthy individuals. Nevertheless, a variety of brain regions have been identified in the regulation of impulsivity or aggression, and some of these structures even appear to contribute to the control of both of these behaviors (Winstanley et al., 2005).

In this respect, the functional network for choice impulsivity is suggested to include medial prefrontal (mPFC) and orbitofrontal cortices (OFC), limbic structures such as nucleus accumbens, hippocampus and amygdala, as well as the mesencephalic dopaminergic system, especially the ventral tegmental area, as well as the dorsal raphe nucleus (DRN) within the brainstem (for review see: Baunez and Lardeux, 2011; Cardinal, 2006; Dalley et al., 2011). All of these brain areas have additionally been associated with aggression control, although several other structures are part of the aggression network as well. These include septum and Bed nucleus of the stria terminalis, which are strongly associated with the amygdala in terms of function and connectivity, or the hypothalamus and the periaqueductal grey which are crucial for the control of attack behavior (Veenema and Neumann, 2007; Ko, 2017; Aleyasin et al., 2018b). Given this wide range of relevant brain regions, the present study focusses on the overlapping structures underlying choice behavior and territorial aggression.

The involvement of several brain regions has been investigated by use of lesion techniques as well as pharmacological manipulations. However, these methods may lead to compensatory effects from other brain regions (see, for example, Winstanley et al., 2005) or they may impair other essential aspects of behavior. Inactivations of the striatum, for example, were shown to disrupt locomotor activity, which made it difficult to distinguish this effect from behavioral changes in impulsivity (Tedford et al., 2015; Magnard et al., 2018).

Other studies have used c-Fos immunoreactivity to detect changes in neuronal activation after the execution of behavioral tasks. Concerning impulsivity measures, a study by da Costa Araújo et al. (2010) used this approach with a focus on forebrain and subcortical structures. The authors found increased c-Fos expression levels in the OFC and the nucleus accumbens core region (AcbC) and concluded that both areas play a role in the evaluation of delay lengths, while only the OFC was involved in the assessment of reward sizes. The processing of delay- and reward-related aspects of behavior are both incorporated as essential components in choice impulsivity (Marshall and Kirkpatrick, 2016). While da Costa Araújo and colleagues used so called adjusting procedures to evaluate each behavioral aspect separately, the present study was designed to extend previous findings and examine choice impulsivity per se by use of a delay-discounting (DD) paradigm.

With territorial aggression, c-Fos induction studies have been more frequently conducted than with choice impulsivity and these have consistently found increased activation levels in association with the expression of offensive aggressive behavior in many brain regions (Halász et al., 2002a; Veening et al., 2005; Veenema and Neumann, 2007; Veenema et al., 2007; Toth et al., 2012; Konoshenko et al., 2013; Hong et al., 2014; Biro et al., 2017). In order to differentiate aggression-specific effects from other social or environmental factors, some of these studies combined aggression testing with additional procedures such as a sensory contact model (no physical contact between opponents during confrontation) (Haller et al., 2006), the investigation of sexual behavior (Veening et al., 2005), different housing conditions (individuals vs. groups) (Toth et al., 2012; Biro et al., 2017) or the exposure to a novel environment (new cage vs. home cage) (Nehrenberg et al., 2013). However, a comparative approach with choice impulsivity has not been conducted so far by means of c-Fos immunohistochemistry.

Hence, the present study first aimed at investigating alterations in neuronal activation patterns in selected brain regions of male DA rats in response to behavioral performance. For this, the rats were initially screened for choice impulsivity based on their preference for delayed rewards, and for territorial aggression according to the expression of offensive behavior against an intruder rat. The brains of individuals with lowest and highest values within the behavioral spectra were chosen to detect the most obvious differences between the distinct behavioral phenotypes. Although our previous work on the relationship between impulsive choice and inter-male aggression in DA rats revealed no direct causal link between both traits (see chapter 2), the current study additionally sought to identify potential overlaps as well as dissimilarities in the neuroanatomical and neurochemical basis underlying these behaviors. With regard to previous findings on increased impulsivity and aggression levels, we expected to see most prominent effects in highly-impulsive and medium-aggressive rats than in individuals with low levels of behavior. Concerning the investigated brain regions, we anticipated a particular involvement of the frontal cortex and the nucleus accumbens in impulsive choice behavior given the connectivity of these structures and that these are important components of the reward circuitry. For aggressive behavior, we assumed additional effects within the basolateral amygdala because of its implication in emotional processes and social behaviors.
Despite possible differences or similarities in the neuroanatomical substrates for impulsivity and aggression, the serotonergic system, and especially the 5-HT_{1A} receptor, was often implicated in the modulation of both behaviors. In particular, pharmacological evidence indicates that activation of this receptor subtype by selective agonists exerts an inhibitory effect on territorial aggression (Mos et al., 1993; Olivier et al., 1995; de Boer et al., 2000; van der Vegt et al., 2003; de Boer and Koolhaas, 2005) and that it can affect impulsive choices, although increased (Evenden and Ryan, 1999; Winstanley et al., 2005; van den Bergh et al., 2006a; Stanis et al., 2008; Blasio et al., 2012) as well as decreased impulsivity levels were reported (Bizot et al., 1999; Zaichenko et al., 2013; Yates et al., 2014). Moreover, a study by Cervantes and Delville (2009) has shown that the number of neurons expressing the 5-HT_{1A} receptor was increased in several brain regions of hamsters with high levels of impulsivity and aggression, but it has to be mentioned that these hamsters were specifically selected for their aggression level. Based on these earlier findings, the second objective of the present study was to assess the involvement of 5-HT_{1A}-expressing neurons in rats that show normal, adaptive levels of impulsivity and aggression.

3.3. Material and methods

3.3.1. Animals

In the present experiment, 18 male Dark Agouti inbred rats (DA/OlaHsd) were used for immunohistochemical analysis following behavioral testing. Behavioral evaluation and analysis of impulsive choice behavior and territorial aggression in DA rats were performed beforehand (see chapter 2). In addition, five DA rats which were not exposed to behavioral paradigms were used as baseline controls in immunohistochemical stainings. DA rats were commercially acquired (ENVIGO, Huntingdon, UK) and bred in our department. As opponents in resident-intruder (RI) tests, 30 male Wistar Han rats were used (Wistar Han IGS, Crl:WI (Han), Charles River, Sulzfeld, Germany; Wistar Han, RccHan^{TM}:WIST, Envigo). Rats of both strains were housed in groups of four to six individuals in Macrolon type IV cages and kept in separate rooms under controlled climate conditions of 21 ± 2°C and 55 ± 5 % humidity on a 12 h light/dark cycle (lights on at 8:00 a.m.). All rats had access to *ad libitum* water and food prior to behavioral testing. At the start of training, DA rats were eight to nine weeks

old and weighed 180 g to 250 g. During operant procedures, they were food restricted and maintained at 85-90 % of the free feeding weight of unrestricted rats. Control rats were 11 to 15 weeks old and had free access to food and water. These rats were directly removed from their home cages for processing of brain tissue. For RI tests, Wistar rats were food restricted to fall below (max. 18 %) or to match the weight of their DA counterparts. Background noise was provided by radio sound in the animal-keeping facility during the light phase and in all test rooms during behavioral testing. Behavioral testing was always performed during the light phase. All experimental procedures were carried out in accordance with the German law on animal protection and the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health, 8th edition, 2011).

3.3.2. Behavioral evaluation

For classification of impulsive choice behavior and territorial aggression, a delay-discounting (DD) task and a RI test were used, respectively. All rats completed both tasks in two different test order groups, such that half of the animals ended with the DD task, while the other half ended with the RI tests. Detailed descriptions of the operant-conditioning apparatus and the procedures are provided in section 2.3.3.

In brief, prior to the DD task, rats underwent four phases of operant training: Habituation to the test chambers (2-3 sessions), lever-press training for left and right levers, nose-poke training for presentation of the levers (12-17 sessions for lever-press and nose-poke training) and a large-reward baseline training (10-23 sessions) for introduction of different reward quantities for each lever (one pellet on the small-reward lever and three pellets on the large-reward lever) and for establishment of preference for the large reward.

The DD task itself was designed after a procedure developed by Evenden and Ryan (1996). Each session consisted of five blocks of 14 trials with four forced-choice trials and 10 free-choice trials. Delays to the large reward increased with session blocks and were set at 0, 10, 20, 40 and 60 s. In between trials, a fixed inter-trial interval of 40 s was chosen. Sessions either terminated after the maximum of 70 trials or 96 min. Each rat received a total of 20 DD sessions. Area under curve (AUC) values were calculated from number of large-reward choices of the final five DD sessions for classification of impulsive choice behavior.

For RI tests, each DA rat was housed with an oviduct-ligated female for six to seven days for establishment of a territory. Residents were tested in their home cages after removal of the female companion, food and water and confronted with an unfamiliar Wistar rat for 10 minutes. Residents performed four RI tests on consecutive days and each test was video-recorded for subsequent analysis of the residents' behavior. The percentage of total aggression time (bite and clinch attacks, lateral threat, chase, keep down, offensive upright) was averaged over all RI tests and individuals were classified based on aggression score classes defined by de Boer and colleagues (2003).

3.3.3. Immunohistochemistry

One hour after the last behavioral test, rats were euthanized using CO_2 and transcardially perfused with PBS and a 4 % paraformaldehyde solution (PFA). Extracted brains were post-fixed in PFA overnight and transferred to a 30 % sucrose solution for cryoprotection until saturation of the tissue. Brains were cut on a cryostat in 40 µm coronal sections and stored in cryoprotectant (30 % glycerol, 30 % ethylene glycol in 0.1 M phosphate buffer) at -30°C until analysis. Sections were sampled into five series with

sections spaced at 200 µm intervals. One series of sections was used for the present study. For immunohistochemistry, free-floating sections were exposed to an antigen retrieval using 0.3 % sodium borohydride in 0.1 M PB, permeabilized with 0.15 % Triton X-100, followed by two blocking steps with BSA (3 %) and goat and donkey normal sera (each 5 %). Sections were incubated for 48 hours with the primary antibodies chicken anti-NeuN (ABN91, 1:1,000), mouse anti-c-Fos (sc-8047, 1:2,000) and rabbit anti-5-HT_{1A} (AB15350, 1:10,000) in a solution containing 0.5 % NGS, 0.5 % NDS and 3 % BSA. These were coupled with the secondary antibodies goat anti-chicken CF405M (20375, 1:2,000), donkey anti-mouse AF594 (150108, 1:600) and goat anti-rabbit AF488 (AP132JA4, 1:800) (each with 0.5 % NGS and 0.5 % NDS), respectively. Subsequently, sections were incubated in 0.3 % Sudan Black B for reduction of lipofuscin particle autofluorescence (also see: Schnell *et al.*, 1999).

3.3.4. Evaluation of immunoreactive cells

Immunoreactive cells were quantified using a triple-fluorescent staining for the neuronal nuclei (NeuN) protein which is specifically expressed in neurons, the c-Fos protein which is a marker for neuronal activation and the serotonin receptor 5-HT_{1A}. For quantification, images were captured unilaterally under a Zeiss Axiophot epifluorescence microscope and cells were counted in three to five sections per brain area according to the size of each area and tissue quality.
Acquired images were processed for several steps using ImageJ software. Firstly, brightness and contrast (B&C) of each image was adjusted automatically. Secondly, threshold values for immunopositive cells were determined manually for each staining and the threshold for one staining was applied to all corresponding images using the auto local threshold option in ImageJ. Overlays of B&C-adjusted images and threshold images were created for cell counts of NeuN-, c-Fos- and 5-HT_{1A}-immunopositive neurons and for double immunostaining of c-Fos and 5-HT_{1A} neurons.
Examined brain areas were the ventral (VO, Bregma +4.6 to +3.8 mm) and lateral orbital (LO, Bregma +4.6 to +3.8 mm), the prelimbic (PrL, Bregma +4.0 to +3.2 mm) and anterior cingulate (Cg, Bregma +2.4 to +1.6 mm) cortices, the nucleus accumbens core (AcbC, Bregma +1.8 to +1.0 mm) and shell (AcbSh, Bregma +2.0 to +1.0 mm), the basolateral amygdala (BLA, Bregma -2.3 to -3.1 mm) and the dorsal (DRD, Bregma -7.3 to -8.3 mm), ventral (DRV, Bregma -7.3 to -8.3 mm) and ventrolateral (DRVL, Bregma -7.3 to -8.3 mm) portions of the dorsal raphe nucleus (DRN; see Fig. 3.1). Sizes of counting areas corresponded to the size of the acquired image and for the frontal cortex regions, the BLA and the nucleus accumbens, the counting frame size was 100.000 μm^2 (acquisition at 200x magnification) and in the DRN, the frame size was 41.500 μm^2 (acquisition at 400x magnification). Counting was performed manually and blind to behavioral group classifications.

3.3.5. Statistical analysis

Cell counts were compared between behavioral groups using IBM SPSS for Windows (version 26.0). A p value < 0.05 was considered significant, and a p value between 0.05 and 0.1 was considered a non-significant trend. All data were initially analyzed for normality using the Shapiro-Wilk test.
Within each brain area, differences between individual rats were analyzed by parametric one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis test with individuals as between-subjects factor and subsequent post hoc multiple comparisons with Bonferroni correction. For analysis of NeuN cell counts, data from one H-Imp rat was excluded for the VO and from one control rat for the

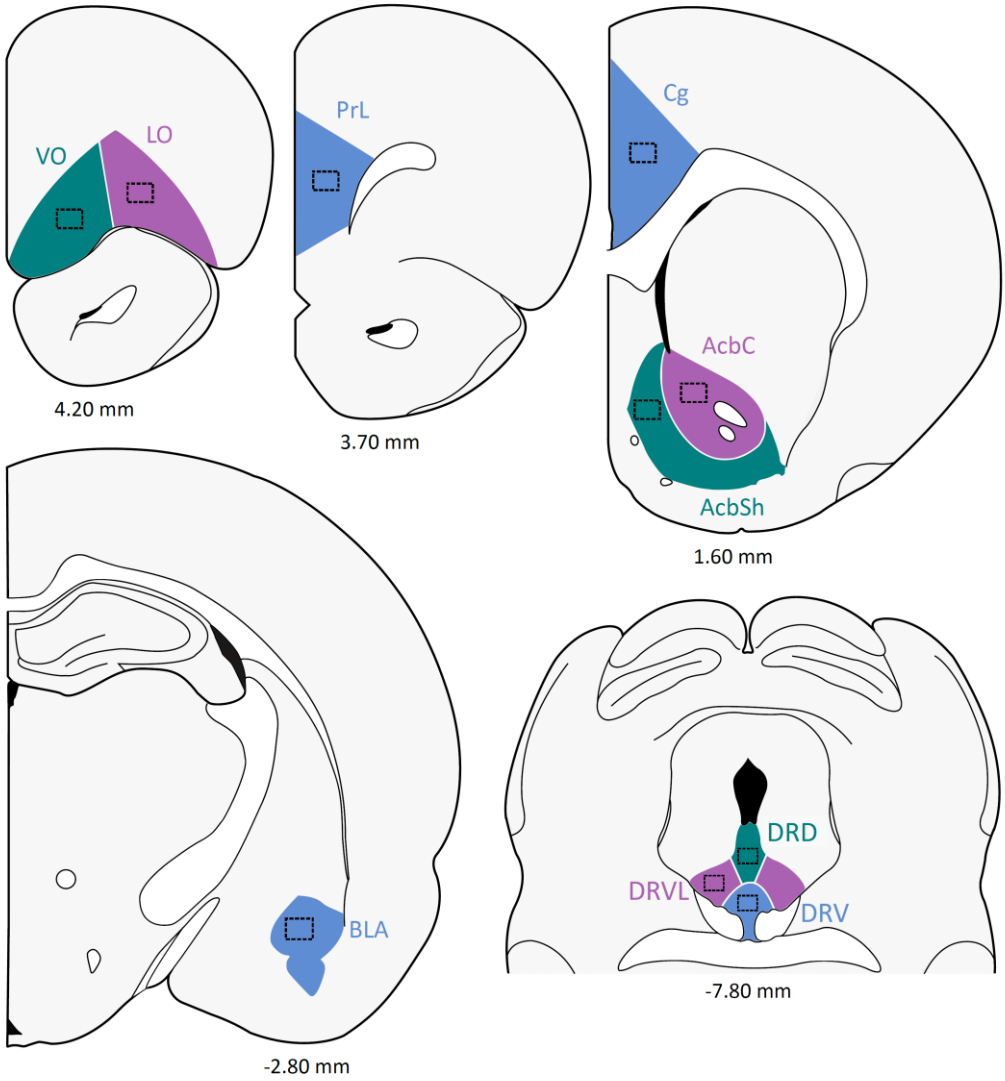

Figure 3.1 | Schematic representation of examined brain regions in the rat brain. The distance from Bregma is shown below each section. Dashed boxes illustrate localizations of acquired photomicrographs for quantification of immunoreactivity. Examined brain regions were the ventral (VO) and lateral orbitofrontal (LO) cortices, prelimbic cortex (PrL) and anterior cingulate cortex (Cg), nucleus accumbens core (AcbC) and shell (AcbSh), basolateral amygdala (BLA), and dorsal (DRD), ventral (DRV) and ventrolateral (DRVL) subregions of the dorsal raphe nucleus. Sections are modified from the rat brain atlas of Paxinos and Watson (1997).

AcbSh due to significant statistical differences between individuals within each behavioral group. Statistics for the comparison of individual rats are treated as initial data for group comparison, which is why these data are not shown.

For each brain area, group comparison was conducted by parametric ANOVA or non-parametric Kruskal-Wallis test with behavioral group as between-subjects factor. Post hoc comparisons with Bonferroni correction were used in both cases to detect detailed group differences. Homogeneity of variance for one-way ANOVA was checked with Levene's test. When this requirement was violated, a Welch test with subsequent Games-Howell post hoc comparisons were used.

3.4. Results

3.4.1. Behavioral data

Rats were classified for impulsive choice behavior and aggression level after performance of a DD task and four RI tests, respectively, as previously described in section 2.3.5. After performance of the DD task, area under curve (AUC) values from the rats' preference curves were computed for classification of choice behavior and individuals that classified as either low- (AUC > 0.67; n = 4) or highly-impulsive (AUC < 0.34; n = 5) were chosen for quantification of immunoreactivity in the present study. From the RI-test group, four individuals displayed an overall low level of aggression (total offensive aggression < 15 %; L-Agg) and these rats did not engage in any aggressive acts in the last RI test. Five individuals were classified as medium aggressive (total offensive aggression 15-55 %; M-Agg) because these rats showed a mean duration of total aggressive behavior of 35.3 % ± 9.0 % (SD). Aggression levels above 55 %, corresponding to a H-Agg phenotype, were not observed in the whole group of rats investigated in chapter 2 of this dissertation. Classification of aggression levels were based on aggression score classes defined by de Boer and colleagues (2003). Behavioral data from both paradigms for individual rats are shown in Table 3.1. The selection of individuals with lowest and highest values for choice impulsivity as well as aggressive behavior was initially done to examine the highest possible differences between groups in terms of neuronal activation assessed by c-Fos protein levels and with regard to potential changes in the number of 5-HT_{1A}-expressing neurons.

Table 3.1 | **Behavioral data for individual Dark Agouti rats following impulsivity and aggression testing.**

Animal #	Final paradigm	Behavioral classification*	AUC value *final DD session*	Offensive aggression [s] *final RI test*
1	DD	L-Imp	**0.96**	0.0
2	DD	L-Imp	**0.83**	19.8
3	DD	L-Imp	**0.79**	21.6
4	DD	L-Imp	**0.77**	0.0
5	DD	H-Imp	**0.17**	0.0
6	DD	H-Imp	**0.15**	5.5
7	DD	H-Imp	**0.12**	0.0
8	DD	H-Imp	**0.11**	8.1
9	DD	H-Imp	**0.08**	0.0
10	RI	L-Agg	0.87	**0.0**
11	RI	L-Agg	0.35	**0.0**
12	RI	L-Agg	0.31	**0.0**
13	RI	L-Agg	0.09	**0.0**
14	RI	M-Agg	0.34	**46.6**
15	RI	M-Agg	0.97	**38.6**
16	RI	M-Agg	0.18	**37.7**
17	RI	M-Agg	0.44	**33.9**
18	RI	M-Agg	0.75	**19.4**

Note. *Classification of impulsive choice behavior is based on mean area under curve (AUC) values from the final five delay-discounting (DD) sessions and aggressive behavior was classified based on the mean duration of total offensive aggression during all four performed resident-intruder (RI) tests. Data from the last conducted tests of each corresponding paradigm are highlighted in bold. H-Imp, highly impulsive; L-Imp, low impulsive; L-Agg, low aggressive; M-Agg, medium aggressive.

3.4.2. Immunohistochemical data

To evaluate possible effects of behavioral measures on neuronal activation and 5-HT$_{1A}$-expressing neurons, selected brain regions were investigated for NeuN-, c-Fos- and 5-HT$_{1A}$-immunoreactivity in the four behavioral groups of rats and the control group. A representative photomicrograph from the ventral orbitofrontal (VO) cortex is shown in Fig. 3.2.

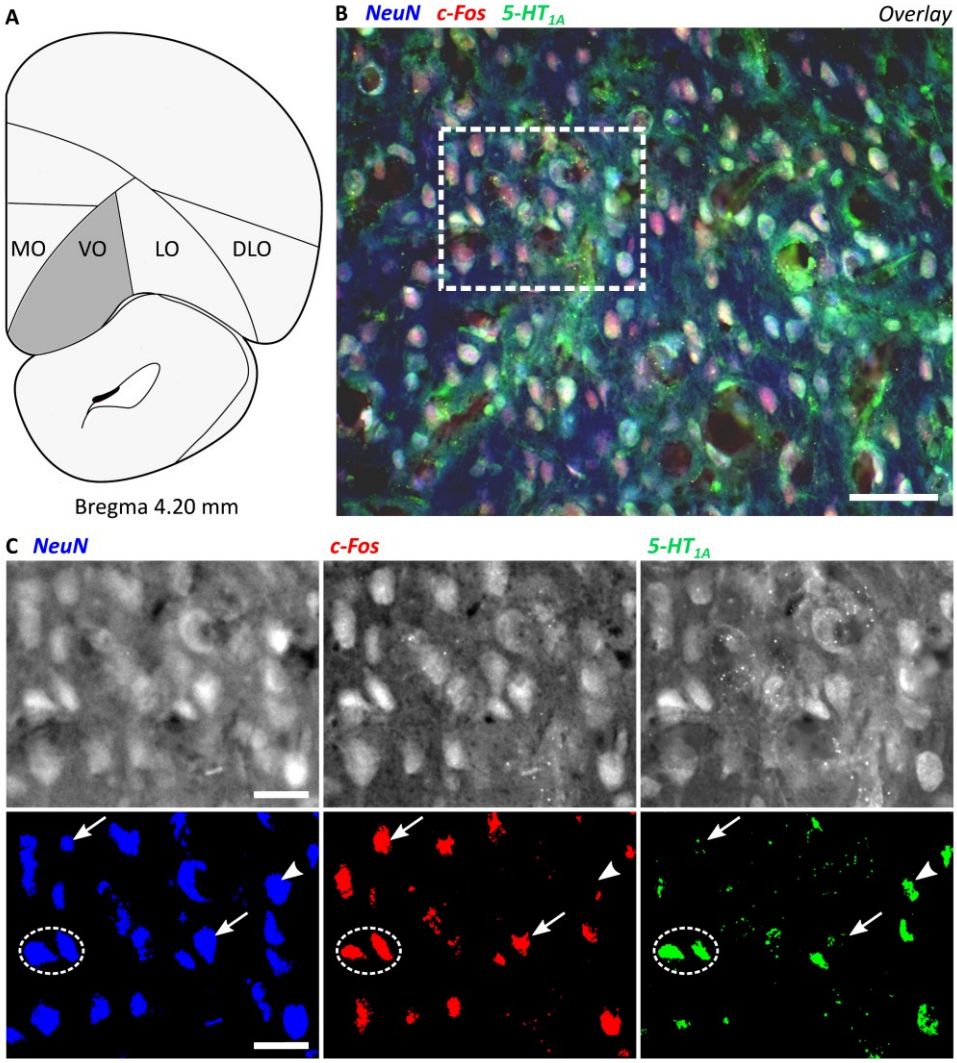

Figure 3.2 | **Immunoreactivity of NeuN (blue), c-Fos (red) and 5-HT$_{1A}$ (green) in the rat ventral orbitofrontal cortex (VO). A**, Schematic drawing of a coronal section at the level of the frontal cortex (Bregma 4.20 mm) from which images in B were captured (shaded region); adapted from Paxinos and Watson (1997). **B**, Representative photomicrograph of the immunohistochemical labeling of NeuN, c-Fos and 5-HT$_{1A}$ shown as a three-channel overlay image. **C**, Single color channels are shown as enlarged selection from B (dashed rectangle) with grayscale images in the upper panel and corresponding threshold images with immunopositive labeling in the lower panel. Arrows, arrowheads and dashed circles illustrate neurons that express immunoreactivity for c-Fos, 5-HT$_{1A}$ and the combination of both, respectively. MO, medial orbitofrontal cortex; LO, lateral orbitofrontal cortex; DLO, dorsolateral orbitofrontal cortex. Scale bars are 50 µm and 20 µm for the overlay image and for enlarged images, respectively.

3.4.2.1. Immunoreactivity in frontal cortex regions

VO. Statistical analysis in the VO revealed highly significant differences between behavioral groups for all stainings (NeuN: $F_{4,56}$ = 7.244, $p < 0.001$; c-Fos: $F_{4,56}$ =12.689, $p < 0.001$; 5-HT_{1A}: $F_{4,56}$ = 4.407, $p < 0.05$; double: H_4 = 14.592, $p < 0.05$). Further multiple comparisons showed a decreased number of NeuN-immunopositive neurons in L-Imp and L-Agg groups each compared to the H-Imp and the control group (Fig. 3.3A). c-Fos expression in this area was decreased for all behavioral groups compared to the control and additionally in L-Agg compared to M-Agg rats (Fig. 3.3B). Additionally, a non-significant trend towards a decreased c-Fos expression in L-Agg compared to H-Imp rats was observed ($p = 0.063$). Post hoc analysis for 5-HT_{1A}-expressing neurons revealed a decreased cell number for L-Imp and L-Agg groups each compared to the control (Fig. 3.3C). Additionally, a trend towards a decrease in 5-HT_{1A}-immunopositive neurons was seen in the L-Imp compared to the H-Imp group ($p = 0.084$). However, differences in 5-HT_{1A}-expressing neurons are similar to differences in NeuN counts and were therefore not further investigated. Neurons labelled for c-Fos and 5-HT_{1A} revealed decreased counts in the VO for L-Agg compared to H-Imp rats and the control group (Fig. 3.3D), which may as well be attributed to differences in NeuN numbers and were excluded from further interpretations.

LO. In the LO, statistical analysis revealed highly significant differences between behavioral groups for c-Fos counts ($F_{4,67}$ = 5.760, $p < 0.001$) and double-labeling ($F_{4,67}$ = 2.641, $p < 0.05$), but post hoc analysis for double-immunopositive neurons revealed no further differences between groups (Fig. 3.3B,D). Multiple comparisons for c-Fos expression revealed a decrease for both aggression groups compared to the control and a trend towards a decrease in the H-Imp group compared to the control ($p = 0.054$). No group effects were observed for NeuN ($F_{4,67}$ = 1.805, $p = 0.138$; Fig. 3.3A) and 5-HT_{1A} ($F_{4,67}$ = 0.842, $p = 0.504$; Fig. 3.3C).

PrL. Statistical analysis in the PrL revealed highly significant differences between behavioral groups only for c-Fos ($F_{4,68}$ = 6.168, $p < 0.001$), while neuron numbers were constant between groups for NeuN ($F_{4,31.783}$ = 1.363, $p = 0.269$), 5-HT_{1A} (H_4 = 7.226, $p = 0.124$) and double-labeling (H_4 = 8.643, $p = 0.071$). Post hoc analysis for c-Fos expression showed a decrease for L-Agg rats compared to H-Imp rats and the control (Fig. 3.3B).

Cg. In the Cg, statistical analysis revealed highly significant differences between behavioral groups for NeuN ($F_{4,69}$ = 5.525, $p < 0.001$), c-Fos (H_4 = 21.78, $p < 0.001$) and double-immunopositive neurons (H_4 = 17.207, $p < 0.05$). No group effects were observed for 5-HT_{1A} (H_4 = 8.195, $p = 0.085$; Fig. 3.3C). Multiple comparisons showed a decreased NeuN number for L-Imp and L-Agg groups compared to the control (Fig. 3.3A). Additionally, a non-significant trend towards a decrease in NeuN in the M-Agg group ($p = 0.062$) was found in comparison to the control. c-Fos expression as well as number of double-labelled neurons in the Cg were decreased in the L-Agg group compared to all other behavioral groups and the control (Fig. 3.3B,D).

Hence, in the frontal cortical regions VO, LO, PrL and Cg, the number of 5-HT_{1A}-expressing neurons was unaltered in all impulsivity and aggression groups. For H-Imp rats, c-Fos expression was decreased in the VO, while for L-Imp rats a similar decrease was found to correspond to differences in NeuN counts. For both impulsivity groups, no further significant differences in c-Fos expression and double-immunolabeling were observed in frontal regions. For both aggression groups, a decreased c-Fos expression was found in VO and LO. However, in the VO, an even lower c-Fos number was observed for L-Agg rats compared to H-Agg rats. Such a difference between aggression groups was also present in the Cg. Additionally, double-immunolabeling was decreased for L-Agg rats in the Cg, whereas in the remaining frontal cortex regions no changes were observed.

3.4.2.2. Immunoreactivity in subcortical limbic structures

NAc. Overall significant group effects were found for all stainings in the NAc core region (NeuN: $F_{4,64} = 21.84$, $p < 0.001$; c-Fos: $H_4 = 37.248$, $p < 0.001$; 5-HT_{1A}: $H_4 = 12.989$, $p < 0.05$; double: $H_4 = 31.923$, $p < 0.001$). In the NAc shell, cell counts differed for NeuN ($F_{4,35.243} = 6.254$, $p < 0.001$), c-Fos ($F_{4,74} = 11.5$, $p < 0.001$) and double-immunostaining ($F_{4,74} = 7.174$, $p < 0.001$), whereas no difference was observed for 5-HT_{1A} ($F_{4,74} = 2.374$, $p = 0.060$). Subsequent multiple comparisons for the core region showed that NeuN counts in all behavioral groups were decreased compared to the control (Fig. 3.3A). Additionally, H-Imp rats had an increased NeuN-positive cell count compared to both aggression groups. Post hoc analysis for c-Fos expression revealed a decreased neuron number in both aggression groups compared to impulsivity groups and control (Fig. 3.3B). Similar group effects were seen for double-immunostaining, i.e., neuron number in both aggression groups was decreased compared to impulsivity groups and control, except for M-Agg and L-Imp groups which showed a trend towards a corresponding decrease ($p = 0.069$; Fig. 3.3D). For 5-HT_{1A}, however, post hoc analysis showed no differences between groups, but trends were observed towards decreased neuron numbers for L-Agg ($p = 0.078$) and M-Agg groups ($p = 0.080$) compared to the control (Fig. 3.3C). In the core region, L-Agg rats had a decreased NeuN number compared to H-Imp rats and the control group (Fig. 3.3A). c-Fos expression as well as double-immunostaining in this region were decreased in the L-Agg group compared to impulsivity groups and the control (Fig. 3.3B,D). The M-Agg group showed a decrease in c-Fos neuron number compared to L-Imp rats and the control and a decrease in double-immunolabeling compared to L-Imp rats.

Thus, in both subregions of the NAc, both impulsivity groups did not differ significantly from each other for all stainings. This was the same for both aggression groups which showed the greatest decrease for c-Fos and double-labelled neurons in both subregions.

BLA. In the basolateral amygdala, overall significant group effects were found for all stainings (NeuN: $H_4 = 26.911$, $p < 0.001$; c-Fos: $F_{4,83} = 22.84$, $p < 0.001$; 5-HT_{1A}: $F_{4,83} = 7.708$, $p < 0.001$; double: $F_{4,83} = 19.21$, $p < 0.001$). Subsequent multiple comparisons revealed a decrease in NeuN-immunopositive cells for all four behavioral groups compared to the control (Fig. 3.3A). This difference was also the case for c-Fos counts and double-immunostaining (Fig. 3.3B,D). However, an even lower decrease was observed for c-Fos expression and double-immunolabeling in the L-Agg group compared to all other groups. Post hoc analysis for 5-HT_{1A}-immunopositive neurons revealed a decrease in neuron number for L-Imp and both aggression groups compared to the control (Fig. 3.3C). Additionally, a trend towards a decreased cell count for the H-Imp group was seen compared to the control ($p = 0.055$).

Thus, except for the low cell counts of L-Agg rats for c-Fos and double-immunostaining, all other group differences for c-Fos, 5-HT_{1A} and double-labeling are similar to differences in NeuN counts and were therefore excluded from further analysis. However, in this brain area, both aggression groups differed significantly from each other regarding c-Fos expression and double-labeling as observed for Cg, and c-Fos expression in the VO.

3.4.2.3. Immunoreactivity in the dorsal raphe nucleus

DRN. In the dorsal raphe nucleus, groups differed significantly by NeuN number in the ventral subregion ($F_{4,64} = 2.735$, $p < 0.05$). However, post hoc analysis revealed no further group differences but a trend towards an increased neuron number in H-Imp rats as compared to the control ($p = 0.054$; Fig. 3.3A). Within dorsal and dorsolateral subregions, NeuN cell counts did not differ between groups

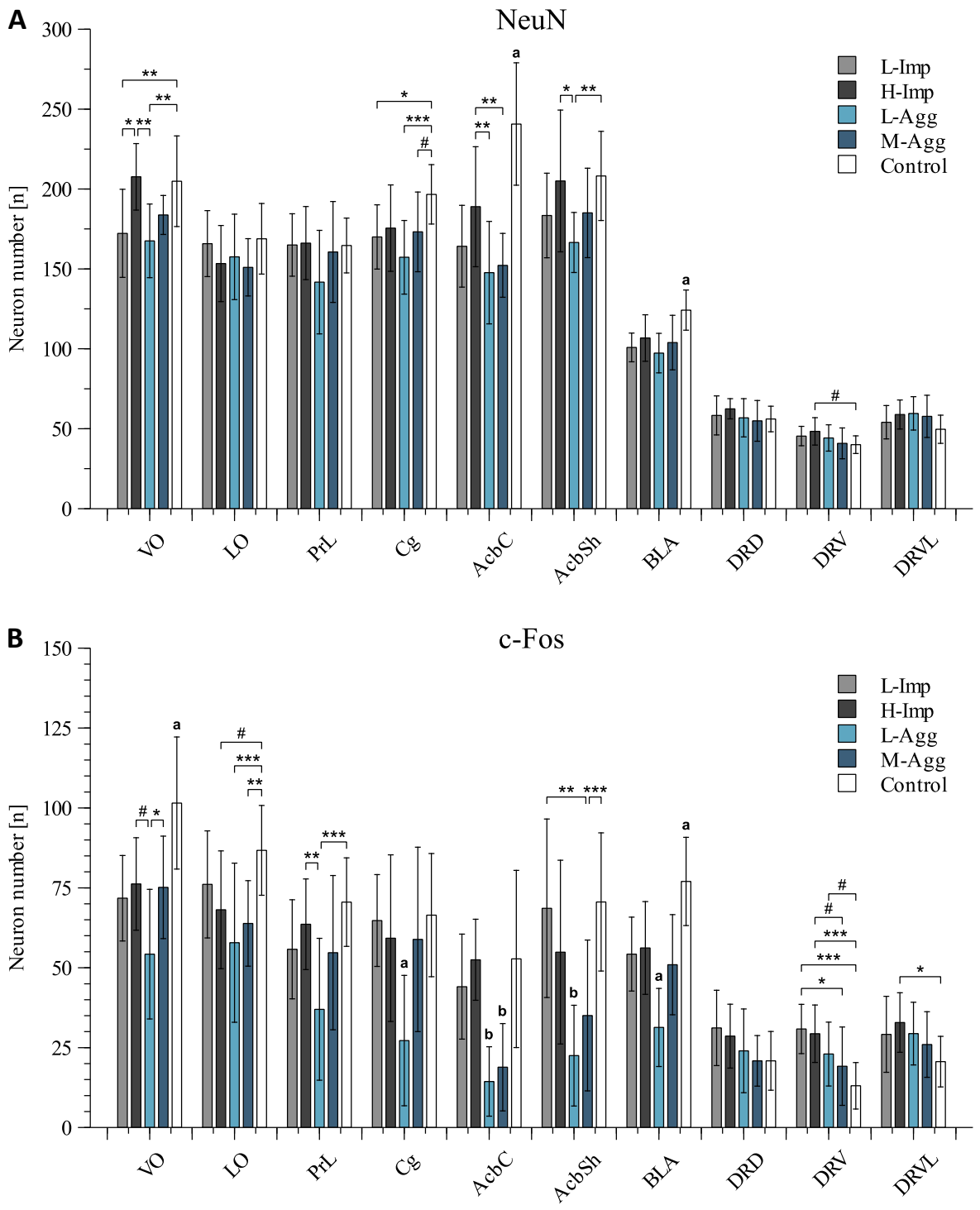

Figure 3.3 | **Number of immunopositive neurons in selected brain regions of low-impulsive (L-Imp), highly-impulsive (H-Imp), low-aggressive (L-Agg) and medium-aggressive (M-Agg) groups of rats and a baseline control group.** Cells were quantified for the expression of neuronal nuclei (NeuN, **A**), c-Fos protein (**B**), the 5-HT_{1A} receptor (**C**) and the double-labeling of c-Fos and 5-HT_{1A} (**D**). Data are expressed as mean ± SD. For abbreviations of brain regions see text. a, significant difference compared to the four remaining groups; b, significant difference compared to L-Imp, H-Imp and control groups; *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.1 (trend).

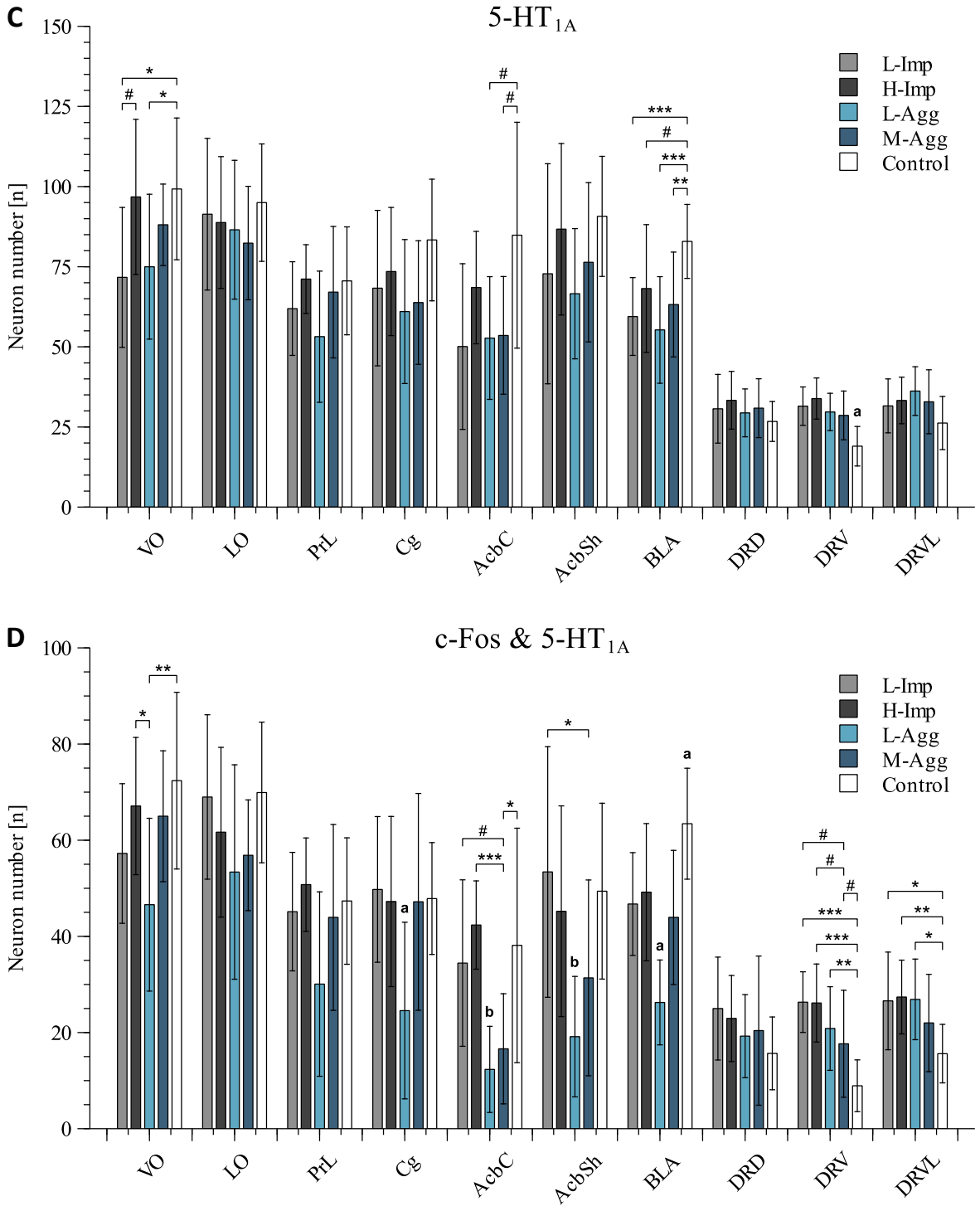

Figure 3.3 (Continued)

(DRD: $F_{4,64}$ = 1.168, p = 0.333; DRVL: $F_{4,60}$ = 1.940, p = 0.115). For c-Fos number, a significant group effect was found in all subregions (DRD: H_4 = 9.71, p < 0.05; DRV: $F_{4,64}$ = 8.331, p < 0.001; DRVL: $F_{4,60}$ = 2.943, p < 0.05). Post hoc analysis for the DRD, however, did not show further significant differences. In the DRV, a significant increase in c-Fos expression was found for both impulsivity groups compared to the control as well as the L-Imp group compared to the M-Agg group (Fig. 3.3B). Moreover, trends towards increased c-Fos expression were found for the L-Agg group compared to the control (p = 0.074) and the H-Imp group compared to the M-Agg group (p = 0.053). In the DRVL, an

increase in c-Fos expression was observed for H-Imp rats compared to the control. Concerning 5-HT_{1A}-immunopositive neurons, groups differed significantly in the ventral subregion ($F_{4,64} = 11.24$, $p < 0.001$) and post hoc analysis revealed a significant increase in all behavioral groups compared to the control group (Fig. 3.3C). Within dorsal and dorsolateral subregions, 5-HT_{1A} cell counts did not differ between groups (DRD: $F_{4,64} = 1.165$, $p = 0.335$; DRVL: $F_{4,60} = 2.282$, $p = 0.071$). Neurons immunopositive for both, 5-HT_{1A} and c-Fos, showed a significant group effect within ventral and ventrolateral subregions (DRV: $F_{4,64} = 10.787$, $p < 0.001$; DRVL: $F_{4,60} = 4.360$, $p < 0.05$). In the DRV, a significant increase in double-labeled neurons was found for both impulsivity groups and L-Agg rats compared to the control (Fig. 3.3D). Moreover, trends towards increased double-labeling were found for the M-Agg group compared to both impulsivity groups (L-Imp: $p = 0.085$; H-Imp: $p = 0.073$) and to the control ($p = 0.051$). In the DRVL, an increase in double-immunopositive neurons was observed for both impulsivity groups and the L-Agg group compared to the control. In the DRD, no significant group effect was found ($H_4 = 9.257$, $p = 0.055$).

Thus, NeuN counts were constant between groups in all DRN subnuclei. In the DRD, no group effects were found for all stainings. In contrast, in the DRV, counts for c-Fos, 5-HT_{1A} and double-labeling were increased for both impulsivity groups. For both aggression groups, increased 5-HT_{1A} counts were observed in the DRV as well as increased double-labeling for L-Agg rats in DRV and DRVL. In the latter subnucleus, increased double-labeling was also observed for both impulsivity groups together with an increased c-Fos expression for H-Imp rats.

3.5. Discussion

The present study investigated the influence of impulsive choice behavior and territorial aggression on neuronal activation patterns, as measured by c-Fos protein expression, and changes in the number of neurons expressing the serotonin 5-HT_{1A} receptor. Several brain areas were examined that have been previously associated with both of these behaviors. With regard to choice impulsivity, changes in neuronal activation were found in the VO subregion of the frontal cortex and in the DRN, while all remaining brain regions were unaffected by DD task performance. Changes in 5-HT_{1A}-expressing neurons were only observed in the ventral subregion of the DRN. After aggression testing, changes in neuronal activation were observed in the frontal cortex, NAc, BLA, as well as the DRN. Except for the DRV, the number of 5-HT_{1A}-expressing neurons per se was unaltered by aggression testing, but changes in double-labeling of c-Fos and 5-HT_{1A} were found in Cg, BLA, NAc and the DRN.

3.5.1. Frontal cortex regions in impulsivity

Performance of the DD task induced changes in the VO, whereas all remaining frontal cortex regions were unaffected by delayed reinforcement. Specifically, H-Imp choice behavior inhibited neuronal activation as compared to control rats without changes in 5-HT_{1A}-expressing or double-labeled cells. The sole decrease in c-Fos expression indicates that non-5-HT_{1A}-expressing neurons were inhibited in the VO in individuals with high delay aversion. Therefore, delay aversion appears to be associated with inhibition of the OFC, specifically the ventral subdivision. This result is in contrast to an earlier study by da Costa Araújo and colleagues who found an increase in c-Fos expression after performance of a DD task in this subregion (da Costa Araújo et al., 2010). In the PrL and the Cg, however, we found no effect induced by choice behavior which is in accordance with findings in the PrL (da Costa Araújo et al., 2010) and with lesion studies in the Cg (Cardinal et al., 2001; Rudebeck et al., 2006).

A great number of studies has shown that the OFC is strongly implicated in impulsive choice behavior (for review see: Baunez and Lardeux, 2011; Cardinal, 2006; Grigoryan, 2012), although findings following lesions of the OFC showed contradictory results with regard to delayed reinforcement. Increased (Mobini et al., 2002; Rudebeck et al., 2006) as well as decreased impulsive choice (Winstanley et al., 2004b; Mar et al., 2011) was found after lesions of the whole OFC, whereas one study reported no influence on this type of impulsivity (Abela and Chudasama, 2013). One factor for varying results might be that lesion sites slightly differed between studies, because some subregions (MO, DLO) were not always included when it is stated that one brain region was lesioned entirely. Mar and colleagues additionally examined selective lesions of LO and MO which increased and decreased impulsivity, respectively (Mar et al., 2011). Decreased impulsivity was also reported after selective VO inactivation (Winstanley et al., 2004b). Therefore, our findings for the OFC, that VO inhibition is associated with increased choice behavior and that the LO is not affected, are in contrast to these earlier findings. Distinct regions of the OFC seem to be diversely involved in the regulation of choice impulsivity, although the precise regional assignment remains to be explored.
Although we found a difference between H-Imp individuals and controls, no difference occurred between impulsivity groups. The present findings might therefore be independent of impulsivity level. The frontal cortex, including OFC and mPFC, is generally involved in goal-directed behavior, cognitive flexibility and reward-related learning (Winstanley et al., 2006a; Schoenbaum et al., 2011; Fitoussi et al., 2015). Specifically, the OFC is thought to play a role in the integration of reinforcer value (Cardinal, 2006; Winstanley et al., 2006b; Fitoussi et al., 2015) as well as maintaining and updating value representation during performance of a task (Schoenbaum et al., 2011; Rudebeck and Murray, 2014), while the mPFC is involved in temporal reward processing (Dietrich and Allen, 1998; Cardinal et al., 2001; Winstanley et al., 2006b; Cassaday et al., 2014) and the Cg specifically processes effort-based decision-making (Walton et al., 2003; Schweimer and Hauber, 2005).
Considering these functional aspects, the sole involvement of the OFC in the present study might be explained by an adaptation of activation levels (Skórzewska et al., 2008; Fitoussi et al., 2015) in the mPFC during earlier processing steps involved in impulsive choice, due to the long-lasting discounting procedure together with preceding operant training. While information on reward contingencies might have been constantly updated by the OFC during the course of the DD task and beyond establishment of stable behavior, processes at earlier stages of the performed task that are regulated by the PrL or the Cg including reward-related learning or timing processes (Cardinal et al., 2001) might have been downregulated beforehand.

3.5.2. DRN in impulsivity

We investigated the DRN with its dorsal, ventral and ventrolateral subnuclei because it constitutes the largest source of the neurotransmitter serotonin (5-HT) (Grigoryan, 2012; Nakamura, 2013) and it contains the serotonin 5-HT_{1A} receptor which acts as an autoreceptor on serotonergic neurons (Mengod et al., 2015).
The DRN was the only investigated brain area with an increase in c-Fos expression in behavioral groups compared to the control. In all other investigated brain areas, c-Fos levels were either decreased or remained unchanged. The highest neuronal activation within the DRN was seen in the ventral subnucleus for rats that performed the impulsivity task and in the ventrolateral subnucleus for H-Imp rats.

Moreover, an increase in 5-HT_{1A}-expressing neurons was observed in the DRV for all behavioral groups relative to control. In both impulsivity groups, this was accompanied by a higher number of double-labelled cells.
Thus, performance of the DD task involved activation of the ventral subnucleus of the DRN and simultaneously up-regulated 5-HT_{1A}-expressing neurons.
In the ventrolateral subnucleus, the number of 5-HT_{1A}-expressing neurons itself was unaltered, but number of double-labeled neurons was enhanced which might indicate that H-Imp choice behavior selectively activated 5-HT_{1A}-expressing neurons in the DRVL.
It was previously shown that receipt of expected as well as unexpected rewards resulted in the activation of DRN 5-HT neurons (Li et al., 2016). Other studies reported that stimulation of these neurons promoted reward waiting (Miyazaki et al., 2014; Luo et al., 2015), in part even without subsequent reward acquisition (Fonseca et al., 2015). Therefore, enhancement of c-Fos expression in the DRN in the present study may be associated with reward- (Nakamura, 2013) and delay-related behavior. Performance of the DD task activated neurons in the DRN regardless of the rat's impulsivity level, because L-Imp and H-Imp groups were not significantly different. A study by Nakamura et al. has shown that different subsets of DRN neurons were activated by the prediction and receipt of either large or small rewards (Nakamura, 2013). A similar increase in neuronal activation of the DRN might therefore arise in individuals with varying behavioral levels as seen in the present study with L- and H-Imp rats. The neuronal activation in DRV and DRVL therefore appears to be linked to different reward contingencies and delay options.
Involvement of the 5-HT_{1A} receptor in choice impulsivity was previously investigated by systemic administration of 5-HT_{1A} agonists which increased choice for a small reward (Winstanley et al., 2005; van den Bergh et al., 2006a). In the DRN, such receptor agonists might inhibit serotonergic neurotransmission via negative feedback mechanisms exerted through 5-HT_{1A} autoreceptors (Celada et al., 2001; de Boer et al., 2009), which were found to be the main type of receptor on serotonergic neurons in the DRN (Mengod et al., 2015). However, in the present study, c-Fos and 5-HT_{1A} expression patterns varied between DRN subregions, but not between impulsivity groups within each region. Thus, it remains unclear how altered expression or activation of the 5-HT_{1A} receptor in DRV and DRVL might contribute to the regulation of impulsive choice behavior in Dark Agouti rats.
Nevertheless, results of the current study show activation of the DRN by a DD task with a regionally specific involvement of ventral and ventrolateral subnuclei, while the dorsal subnucleus was not influenced by this task.

3.5.3. Impulsivity, interactions of brain regions and subcortical areas

Taken together, three out of 10 examined brain regions were affected by the performance of the DD task in male Dark Agouti rats. These include ventral and ventrolateral parts of the DRN and the ventral orbitofrontal cortex.
The OFC and DRN are interconnected brain regions which are both associated with reward-related aspects of behavior (Winstanley et al., 2006b; Muzerelle et al., 2016). Given this interconnectivity and functional similarities, present data might to some extent demonstrate an interaction between both of these areas in the performance of delayed reinforcement. However, since the current study did not directly analyze connectivity measures, a relationship between neuronal activation patterns in the DRN and the OFC is highly speculative. One example for the analysis of specific pathways involved in choice behavior was recently shown by Meda and colleagues who combined a DD task with a tracing technique and a specific cell extraction method (laser capture microdissection) to identify receptor

mRNA expression profiles of traced neurons (Meda et al., 2019). With this approach, the authors identified that increased choice impulsivity was associated with 5-HT_{1A} receptor mRNA levels in pathways originating in OFC and PrL and projecting to the BLA. Moreover, the latter study reported an association of different dopaminergic receptors with cortical projections to the NAc, which is only one example for the involvement of the dopaminergic system in delay discounting (for review see: Jupp and Dalley, 2014; Winstanley, 2011).

In the present study, neuronal activation levels of the BLA and the NAc were unaffected following the DD task. This is in contrast to previous studies which reported an increased c-Fos protein level in the NAc induced by delayed reinforcement (da Costa Araújo et al., 2010), or which repeatedly found that excitotoxic lesions of both areas enhanced choice impulsivity (Cardinal et al., 2001; Winstanley et al., 2004b; Pothuizen et al., 2005; Bezzina et al., 2007; Churchwell et al., 2009; da Costa Araújo et al., 2009; Galtress and Kirkpatrick, 2010; Valencia-Torres et al., 2012; Feja et al., 2014). One possible explanation may be that neuronal activation of BLA and NAc neurons differ during the course of the DD task, as already discussed for frontal cortex regions (see section 3.5.1.). Accordingly, in the present study, changes in neuronal activation patterns might have not been detected potentially due to an adaptation of activation levels (Skórzewska et al., 2008; Fitoussi et al., 2015).

3.5.4. Aggression and the frontal cortex

Performance in inter-male confrontations induced neuronal inhibition in all examined frontal cortex regions (VO, LO, PrL, Cg) in L-Agg rats as well as in both orbitofrontal regions (VO, LO) in M-Agg rats. Immunostainings for 5-HT_{1A} and the double-labeling of c-Fos and 5-HT_{1A}, however, were unaffected, except for the Cg, in which double-labeling was decreased. These results indicate an inhibition of 5-HT_{1A} neurons in the Cg and non-5-HT_{1A} neurons in VO, LO and PrL, as found in the present study for H-Imp rats in the VO. Neuronal inhibition was thereby most prominent for L-Agg rats in all frontal cortex regions relative to all other groups. Moreover, a significant difference between aggression groups was present in VO and Cg indicating that these regions are specifically affected by aggression level, which was not observed in LO or PrL.

Several frontal cortex regions were previously shown to play a role in aggressive behavior (Halász et al., 2006; Miczek et al., 2015), although diverse relations were found. Increased c-Fos counts were reported for aggressive individuals in VO, LO (Toth et al., 2012), mPFC (PrL & IL) (Biro et al., 2017), PrL and Cg (Toth et al., 2012), indicating a similar link between aggression and neuronal activation in OFC and mPFC as found in the present study although the direction of activation patterns and behavioral levels differed. In contrast, optogenetic activation of the mPFC was reported to decrease aggression (Takahashi et al., 2014), while mice selected for L-Agg behavior showed unchanged c-Fos expression levels in this area (Haller et al., 2006). In the OFC, optogenetic activation had no effect (Takahashi et al., 2014), but lesioning of this area was reported to increase aggression (Rudebeck et al., 2007). The latter finding may be comparable to OFC inhibition in M-Agg rats of the present study, at least for the VO subregion, due to significant differences between aggression groups.

For the Cg, selective lesions were reported to have no influence on aggression, but affected social behavior as assessed by a social interaction test (Rudebeck et al., 2007). Actually, aggressive behaviors expressed during inter-male confrontations are only one aspect of social communication between conspecifics (Takahashi and Miczek, 2014; Tulogdi et al., 2015; Wrangham, 2018). In a study by Halász and colleagues, for example, c-Fos expression levels were increased in several frontal cortex regions by both inter-male confrontations and psychosocial contacts. During the latter approach, resident rats were separated from their opponents to prevent physical contacts, while olfactory and visual

inspections were possible (Halász et al., 2006). The authors suggested that these activation patterns were induced by social arousal rather than aggressive behavior. Hence, given that aggression groups of the present study did not differ significantly in LO and PrL, neuronal inhibition in these areas might have been associated with social arousal, while involvement of VO and Cg appears to be aggression-specific.

3.5.5. Aggression and the BLA

Differences in neuronal activation between both aggression groups were additionally observed in the BLA in which L-Agg rats showed the lowest activation level, as already observed in frontal cortex regions. This was accompanied by a reduced double-immunostaining indicating inhibition of 5-HT_{1A}-expressing neurons.
Previous studies found that engagement in aggressive behavior induced c-Fos expression in the BLA in the rat (Veening et al., 2005; Toth et al., 2012; Biro et al., 2017), whereas in mice repeated winning experience reduced c-Fos levels (Smagin et al., 2015).
These findings suggest an involvement of the BLA in the regulation or execution of aggression rather than its inhibition as observed in L-Agg individuals in the present study. Nevertheless, c-Fos expression levels differed between both aggression groups and thus appear to be aggression-specific.

3.5.6. Aggression and the NAc

The most prominent decrease in c-Fos expression and in number of double-labeled neurons was observed in the NAc for both aggression groups. This expression pattern is similar to the one found in Cg and BLA indicating inhibition of 5-HT_{1A}-expressing neurons, but in the NAc, aggression groups did not differ significantly from each other. Thus, we suggest that inter-male confrontations induced an inhibition of AcbC and AcbSh regions which was not aggression-specific. Moreover, among all brain areas investigated, the NAc was the only one for which both aggression groups differed compared to all other groups.
An implication of the NAc in aggression was also shown in previous studies which examined neuronal activation as well as specific contributions of the serotonergic and dopaminergic system by agonistic encounters. Fight-induced increase in c-Fos levels were reported for the AcbSh, whereas the AcbC was unaffected (Toth et al., 2012). Additionally, decreased 5-HT levels were found in the NAc prior to agonistic confrontations, while dopamine levels increased at this point, lasting until the separation of resident and intruder (van Erp and Miczek, 2000; Ferrari et al., 2003). Another study found an enhanced number of 5-HT_{1A} neurons in H-Agg compared to L-Agg hamsters (Cervantes and Delville, 2009). Although in the current study 5-HT_{1A} neuron number itself was unaltered, changes in double-immunoreactivity indicate inhibition of these neurons, which might support previous findings of an involvement of the NAc in inter-male confrontations mediated via the serotonergic system.

3.5.7. Aggression and the DRN

The DRV also seemed to play a role in inter-male confrontations because the number of 5-HT_{1A} neurons was enhanced in both aggression groups. This was accompanied by increased double-labeling in L-Agg rats, while neuronal activation levels were unaltered in both aggression groups. The latter finding is in line with a previous study which found no influence of aggressive acts on c-Fos expression in the whole

DRN (Toth et al., 2012). In contrast, other studies reported increased c-Fos expression in H-Agg subjects after RI tests (van der Vegt et al., 2003; Mark et al., 2019). Moreover, pharmacological manipulations of DRN neurons by a 5-HT_{1A} receptor agonist inhibited serotonergic neurotransmission and diminished aggressive behavior (van der Vegt et al., 2003). A similar link was found in another case, in which aggression in RI tests increased 5-HT neurotransmission (Mark et al., 2019).
Present results support an involvement of the DRV in inter-male confrontations via the serotonergic system, although neuronal activation was unaffected. Thus, an increase in 5-HT_{1A}-expressing neurons might have inhibited 5-HT neurotransmission via negative feedback mechanisms exerted through 5-HT_{1A} autoreceptors (Celada et al., 2001; de Boer et al., 2009). This in turn may have inhibited aggressive behavior in L-Agg rats, being in line with reported anti-aggressive effects of 5-HT_{1A} agonists (de Boer et al., 2000; van der Vegt et al., 2003). However, given that aggression groups did not differ significantly in the number of 5-HT_{1A}-expressing neurons and that a trend towards increased double-labeling in M-Agg rats was observed, effects on the DRV appear to be independent of aggression level. Changes in the number of 5-HT_{1A}-expressing neurons in this subregion might as well be associated with social arousal, as previously discussed for neuronal activation patterns in frontal cortex regions (see section 3.5.1.). Earlier investigations in the DRN have shown that neuronal activation in the entire raphe nucleus was induced by both aggression and psychosocial contacts (Haller et al., 2005). In the same study, however, a reduction in aggressive behavior was associated with increased activation of DRN 5-HT neurons specifically. Accordingly, activation of the whole DRN appears to exert different effects on aggression compared to the specific activation of 5-HT neurons (Haller et al., 2005), suggesting that aggressive behavior may be controlled by different subpopulations of DRN neurons. Although in the present study, an increased availability of the 5-HT_{1A} receptor indicates an inhibition of putative 5-HT neurons in both aggression groups, the observed changes in 5-HT_{1A}-expressing neurons generally demonstrate an involvement of the DRV serotonergic system in inter-male confrontations.
In summary, current results and earlier studies revealed contradictory associations of the serotonergic system with aggressive behavior performed in RI tests. While for maladaptive forms of aggression the general serotonin deficiency hypothesis postulates that low levels of serotonin are associated with high levels of aggression (for review see: de Boer and Koolhaas, 2005), findings on adaptive forms of aggression have linked enhanced serotonin expression to high levels of aggression (de Boer et al., 2009; Mark et al., 2019). Current results, however, cannot clearly confirm or reject one of these hypotheses.

3.5.8. Baseline control levels

One caveat of the present study is that the number of immunopositive neurons for the c-Fos staining was highest in the baseline control group compared to behavioral groups, at least in most brain areas investigated. As a consequence, significant differences to impulsivity or aggression groups are interpreted as neuronal inhibition induced by the corresponding behavior or task performance.
In other studies which used a similar method to identify neuronal activation patterns following behavioral testing, control groups mostly displayed the lowest level of activation such that behavioral performance either induced activation or had no effect (Haller et al., 2005; Veening et al., 2005; Toth et al., 2012; Fitoussi et al., 2015; Biro et al., 2017). For example, in the study by Biro et al., aggressive behavior, as assessed by RI tests, increased neuronal activation compared to the control, whereas in the present study, aggressive behavior mainly resulted in decreased neuronal activation. However, in one brain area investigated in the present study, i.e., the DRV, a trend towards an increase in neuronal activation was observed after RI tests, which might indicate that baseline control levels in DA rats are

as high as observed in the present study. Another factor for varying baseline levels might be the type of control with regard to housing or treatment conditions. In the present study, rats of control and behavioral groups were likewise housed in groups of four to six animals. However, this was changed for aggression testing, because residents were housed with a female companion seven days prior to and during RI tests. Moreover, for operant training and the subsequent DD paradigm, DA rats were food deprived. For each of these conditions, it is possible to use a corresponding control group to provide the best conditions for comparison and to control for potential confounding factors. For instance, previous studies have used control groups that were housed with a female without performance of RI tests (Veening et al., 2005) or which had been food-deprived without exposure to an operant task (Fitoussi et al., 2015). For the present study, an undisturbed cage control was chosen as a baseline control for immunostainings to provide a basis for both behavioral paradigms which differ in their preparatory phases. Examples for undisturbed controls (Haller et al., 2005) or housing conditions which are similar to the ones used in the present study (Biro et al., 2017) are found as well, but in all of these studies activation levels of controls were fairly low. However, group-housed and isolated rats were previously found to not differ in their c-Fos expression levels (Toth et al., 2012), which might indicate a minor influence of the control condition on behavioral outcome. Hence, the reason for the somewhat high neuronal activation levels of control rats of the present study remains unresolved. Nevertheless, irrespective of the direction of effects, present results have shown that the performance in a DD task and in RI tests induced changes in c-Fos expression in several brain regions, thus providing additional evidence for the involvement of these brain regions in behavioral control.

3.5.9. Conclusion

To summarize, in male DA rats, the performance of a DD task was associated with inhibition of the OFC, specifically the VO, and with activation of the DRN, specifically ventral and ventrolateral subregions. In two of these areas (VO, DRVL), effects were observed in individuals with increased choice impulsivity, while DRV activation was independent of impulsivity level.

With regard to aggression, all brain regions investigated were involved in RI performance, except for DRD and DRVL. Moreover, present results always revealed an inhibition of involved brain areas. Aggression-specific effects were observed in VO, Cg and the BLA with lowest c-Fos expression levels in L-Agg individuals. This might indicate that neuronal inhibition in these areas is necessary to suppress aggressive behavior.

Accordingly, present results show little overlap of involved brain regions between choice impulsivity and inter-male, territorial aggression. Such a relation was only observed in the VO, which was inhibited in H-Imp as well as L- and M-Agg rats. Hence, both behavioral traits appear to be mainly controlled by different brain regions, and thus have a different neuroanatomical basis.

Investigations of the serotonin 5-HT_{1A} receptor only revealed changes in the DRV, such that the receptor was up-regulated in all behavioral groups. Although this finding additionally links both behavioral paradigms, there is no specific effect of behavior. Thus, the 5-HT_{1A} receptor appears to be involved in more general behavioral processes associated with the performance of both delayed reinforcement and inter-male confrontations.

Chapter 4

Differential changes in dopamine D2 receptor and serotonin 5-HT_{1A} receptor expression in the brains of Dark Agouti rats trained in a delay-discounting task

Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

4.1. Abstract

Serotonin and dopamine are important modulators of various cognitive and physiological functions, including the regulation of impulsive behavior, and by acting on distinct monoaminergic receptors, they may contribute to individual variation in choice impulsivity. In rodents, pharmacological studies have identified the serotonin 5-HT_{1A} and dopamine D2 receptors as crucial receptor subtypes involved in these modulatory processes. By using a delay-discounting (DD) procedure, we previously found that male Dark Agouti inbred rats show a wide range of individual differences for impulsive choice behavior, defined as the tendency to choose a small, immediate reward over a larger, but delayed reward. The present study investigates 5-HT_{1A} and D2 receptor expression in low- (L-Imp) and highly-impulsive (H-Imp) rats by use of immunohistochemical stainings following DD performance. The number of immunoreactive neurons and the regional density of both receptors were determined in selected regions of the frontal cortex, limbic, subcortical structures and brain regions synthesizing serotonin and dopamine.

Expression levels of the 5-HT_{1A} receptor were specifically reduced for L-Imp individuals within the anterior cingulate cortex (Cg), whereas an up-regulation was found in the nucleus accumbens shell (AcbSh), the ventral tegmental area and the dorsal raphe nucleus for both impulsivity groups. The observed increases in 5-HT_{1A} expression, however, were not linked to a particular impulsivity level.

For the D2 receptor, impulsivity-specific effects were observed for H-Imp individuals only, with decreased expression levels in the prelimbic cortex and AcbSh. Additional changes not related to impulsivity level were present in the ventral orbitofrontal cortex and the nucleus accumbens core. These results indicate a regionally specific involvement of both receptors in choice impulsivity, where diminished 5-HT_{1A} activity potentially promotes self-control, while reduced D2 levels may contribute to delay aversion.

4.2. Introduction

The monoaminergic neurotransmitters serotonin and dopamine are involved in a wide variety of brain functions such as cognitive processes, reward, motivation, stress and anxiety, social behavior, locomotion, as well as learning and memory (Björklund and Dunnett, 2007b; Nichols and Nichols, 2008; Beaulieu and Gainetdinov, 2011; Olivier, 2015). Moreover, both transmitter systems have long been associated with the regulation of impulsive behavior, which includes choice impulsivity defined as the inability to delay gratification or an increased preference for immediate versus delayed rewards (Evenden, 1999; Dalley and Roiser, 2012).

There is also some evidence for an involvement of noradrenaline, another monoaminergic neurotransmitter, as demonstrated by pharmacological interventions (for reviews see: Jupp and Dalley, 2014; Pattij and Vanderschuren, 2020). However, given the well-established role and

importance of the serotonergic and dopaminergic systems in this type of impulsivity, the noradrenergic system will not be covered in the present study.

The functions mentioned above are mediated via a large number of dopaminergic and serotonergic receptors located in brain regions that are relevant for the regulation of impulsivity (Cardinal, 2006; Dalley et al., 2011). While serotonin can act on 14 receptor subtypes from seven families, two receptor families with five subtypes have been identified for the dopaminergic system (Barnes and Sharp, 1999; Millan et al., 2008; Beaulieu and Gainetdinov, 2011). In the regulation of choice impulsivity, an important role has been determined for the 5-HT_{1A} and D2 receptors, as pharmacological manipulations of these subtypes were shown to alter choice behavior in delay-discounting (DD) paradigms (see below).

In particular, activation of 5-HT_{1A} receptors by selective agonists was often shown to increase impulsive choices when given systemically (Evenden and Ryan, 1999; Winstanley et al., 2005; van den Bergh et al., 2006a; Stanis et al., 2008; Blasio et al., 2012). A few studies, however, observed the opposite effect with low doses of these agents, namely decreased choice behavior (Bizot et al., 1999; Zaichenko et al., 2013). Depending on the specific receptor localization, 5-HT_{1A} can either function as presynaptic autoreceptor on serotonergic neurons or as postsynaptic heteroreceptor at serotonergic projection sites. Systemic infusions of 5-HT_{1A} compounds might thus simultaneously activate hetero- and autoreceptors in different brain regions and result in contrasting behavioral outcomes (de Boer and Koolhaas, 2005; van den Bergh et al., 2006a). One study by Yates et al. (2014) found that the local administration of a 5-HT_{1A} agonist into the orbitofrontal cortex (OFC) reduced choice impulsivity, whereas medial prefrontal cortex (mPFC) infusions had no effect. This indicates an involvement of the frontal cortex in the modulation of choice behavior via 5-HT_{1A} receptor activity, but with differential roles for distinct cortical subregions.

Regarding the dopamine D2 receptor, pharmacological interventions within OFC and mPFC were previously found to increase impulsive choices, although this was observed with receptor activation (Yates et al., 2014) as well as with receptor blockade (Zeeb et al., 2010; Pardey et al., 2013; Yates et al., 2014). Increased levels of impulsivity were additionally linked to diminished D2/3 receptor availability within the nucleus accumbens (NAc) (Barlow et al., 2018), while low levels of impulsivity were associated with high D2 mRNA expression within the mPFC (Simon et al., 2013). These findings therefore implicate D2 receptor function in cortical and limbic, subcortical areas in this form of impulsive decision-making. However, other pharmacological studies complement these findings. For instance, local infusion of a D2 agonist and a D2 antagonist into the NAc had no effect on delayed choices (Yates and Bardo, 2017), similar to the systemic administration of such compounds (Evenden and Ryan, 1996; Koffarnus et al., 2011; Pattij et al., 2014; Li et al., 2015; Tian et al., 2019). This is also consistent with a recent investigation on D2 receptor mRNA levels which were unaffected by behavioral performance of a DD task in different cortico-amygdala and cortico-striatal pathways (Meda et al., 2019). Hence, the precise role of 5-HT_{1A} as well as D2 receptors in the modulation of impulsive responses is still difficult to establish.

To further examine how different monoaminergic receptors are related to individual levels of impulsive choice behavior, the present study investigates alterations in the expression of 5-HT_{1A} and D2 receptors within selected brain regions of male Dark Agouti (DA) rats. Impulsive behavior in these rats was initially assessed in a DD task, revealing a high range of individual differences (see chapter 2). Accordingly, low- and highly-impulsive groups of rats were selected for analysis in the present study.

As stated above, earlier studies that examined effects for 5-HT_{1A} and D2 receptors within specific brain regions, have mainly focused on the frontal cortex and the NAc. These brain regions are important components of the functional network for impulsivity (Cardinal, 2006; Dalley et al., 2011) and it was

shown that both receptor subtypes are highly expressed within these structures (Aznar et al., 2003; Beaulieu and Gainetdinov, 2011). Other areas with high expression levels include amygdala, hippocampus and hypothalamus (Barnes and Sharp, 1999; Aznar et al., 2003; Beaulieu and Gainetdinov, 2011; Nakamura, 2013), which, along with the frontal cortex and the striatum, constitute parts of the so called limbic corticostriatal loop. This circuitry is highly relevant for the processing of reward-related behavior (Winstanley, 2007; Roesch and Bryden, 2011; Zhang, 2020). Moreover, all of these brain regions receive serotonergic input from the raphe nuclei within the brainstem and dopaminergic input originating in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), i.e., the midbrain dopaminergic system (Björklund and Dunnett, 2007a; Ogawa et al., 2014; Muzerelle et al., 2016). Thus, considerable levels of the 5-HT_{1A} receptor can be found in the raphe nuclei and of the D2 receptor in VTA and SNc, where they each function as autoreceptors to regulate transmitter synthesis and release (Beaulieu and Gainetdinov, 2011; Mengod et al., 2015). Accordingly, in the current study, potential changes in receptor expression are analyzed within cortical and subcortical areas, as well as the dorsal raphe nucleus and both midbrain dopaminergic subregions. For this, we chose an immunohistochemical approach following behavioral performance to examine two measures of receptor expression, namely the number of 5-HT_{1A}- and D2-expressing neurons, on the one hand, and receptor densities independent of neuron number, on the other hand.

4.3. Material and methods

4.3.1. Animals

Subjects were nine male Dark Agouti inbred rats (DA/OlaHsd) which were commercially acquired (ENVIGO, Huntingdon, UK) and bred in our department. For the present study, DA rats were used for immunohistochemical analysis following evaluation of impulsive choice behavior. Prior to impulsivity testing, these rats additionally underwent aggression testing which was performed as part of chapter 2 of this dissertation. Since the present study focusses on impulsive choice behavior, the data on aggressive behavior is not included. An additional group of five DA rats was not exposed to behavioral tests and served as baseline controls in immunohistochemical stainings. Control rats were 11 to 15 weeks old and had free access to food and water. These rats were the same as in chapter 3 of the present dissertation, but a different series of brain sections was used for analysis. Rats were kept on a 12 h light/dark cycle (lights on at 8:00 a.m.) under controlled climate conditions of 21 ± 2°C and 55 ± 5 % humidity and housed in groups of four to six individuals in Macrolon type IV cages. Prior to behavioral testing, all rats had access to *ad libitum* water and food. At the start of operant training, DA rats were eight to nine weeks old and weighed 180 g to 250 g. They were food restricted during operant procedures and maintained at 85-90 % of the free feeding weight of unrestricted rats. In order to provide background noise, radio sound was turned on in the animal-keeping facility during the light phase and in the test room during behavioral testing. Operant procedures were always performed during the light phase. All experimental procedures were carried out in accordance with the German law on animal protection and the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health, 8th edition, 2011).

4.3.2. Evaluation of impulsive choice behavior

Rats were classified for choice impulsivity using a delay-discounting paradigm which was completed after rats were exposed to aggression testing. Detailed descriptions of the operant-conditioning apparatus and the procedures are provided in chapter 2 of the present dissertation.
In brief, rats underwent four phases of operant training prior to the DD task, which included the habituation to the test chambers (2-3 sessions), lever-press training for left and right levers, nose-poke training for presentation of the levers (12-16 sessions for lever-press and nose-poke training) and a large-reward baseline training (10-23 sessions) for the introduction of different reward quantities assigned to each lever (One pellet on the small-reward lever and three pellets on the large-reward lever) and the establishment of a preference for the large reward. The DD paradigm was designed after a procedure developed by Evenden and Ryan (1996). DD sessions consisted of 14 trials with four forced-choice trials and 10 free-choice trials in five session blocks. Delays to the large reward increased with session blocks and were set at 0, 10, 20, 40 and 60 s. A fixed inter-trial interval of 40 s was presented in between the trials. Each rat completed a total of 20 DD sessions. For evaluation of impulsive choice behavior, the area under the curve (AUC) was calculated from number of large-reward choices of the final five DD sessions.

4.3.3. Immunohistochemistry

For immunohistochemical quantifications, rats were euthanized one hour after the last behavioral test using CO_2 and transcardially perfused with PBS and a 4 % paraformaldehyde solution (PFA). After post-fixation overnight in PFA, extracted brains were transferred to a 30 % sucrose solution for cryoprotection until saturation of the tissue. Coronal sections at 40 µm thickness were prepared using a cryostat and stored in cryoprotectant (30 % glycerol, 30 % ethylene glycol in 0.1 M phosphate buffer) at -30°C until analysis. Five series of sections were prepared with sections spaced at 200 µm intervals. A different series of sections than those described in chapter 3 of the present dissertation was used in the present experiments. For immunohistochemical stainings, free-floating sections were exposed to two antigen retrieval steps using 0.3 % sodium borohydride in 0.1 M PB and 1 % sodium dodecyl sulfate in 0.1 M PB. Sections were then permeabilized with 0.15 % Triton X-100, followed by two blocking steps with BSA (3 %) and goat and donkey normal sera (each 5 %). Primary antibodies were chicken anti-NeuN (ABN91, 1:1,000), mouse anti-D2 (sc-5303, 1:1,000) and rabbit anti-5-HT_{1A} (AB15350, 1:10,000) in a solution containing 0.5 % NGS, 0.5 % NDS and 3 % BSA. Secondary antibodies were goat anti-chicken CF405M (20375, 1:2,000), donkey anti-mouse AF594 (150108, 1:2,000) and goat anti-rabbit AF488 (AP132JA4, 1:800) (each with 0.5 % NGS and 0.5 % NDS), respectively. Primary antibodies were applied for 48 hours and secondary antibodies were incubated overnight. Finally, 0.3 % Sudan Black B was used to reduce lipofuscin particle autofluorescence (also see: Schnell *et al.* 1999).

4.3.4. Evaluation of immunoreactive cells

Microscopic images for quantification of NeuN-, D2- and 5-HT_{1A}-immunoreactivity were captured unilaterally under a Zeiss Axiophot epifluorescence microscope. Determined parameters were number of immunopositive neurons for all stainings and receptor density for D2 and 5-HT_{1A} receptors using the percentage of immunopositive pixels within each microscopic image. Three to five sections per brain area were analyzed according to the size of each area and tissue quality.

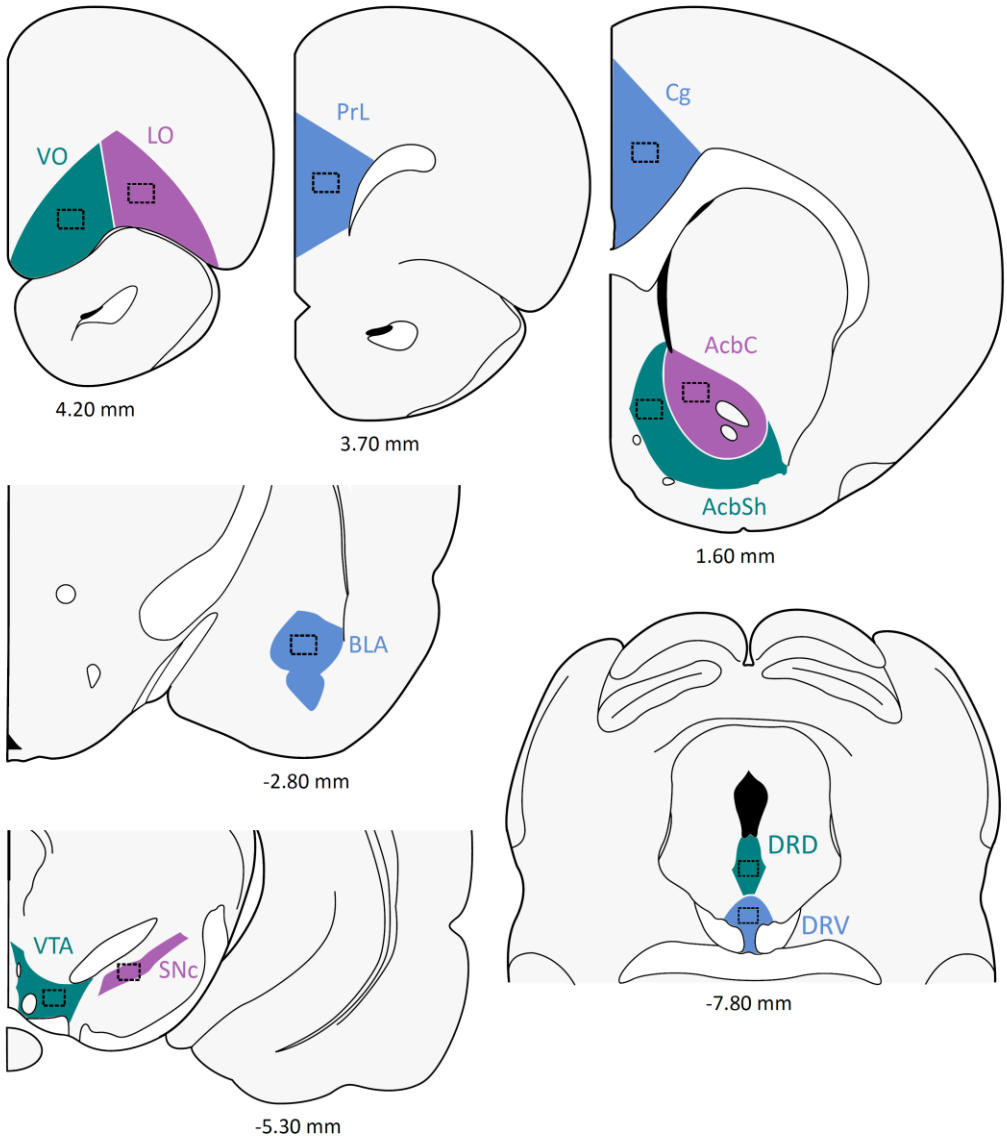

Figure 4.1 | Schematic representation of examined brain regions in the rat brain. The distance from Bregma is shown below each section. Dashed boxes illustrate localizations of acquired photomicrographs for quantification of immunoreactivity. Examined brain regions were the ventral (VO) and lateral orbitofrontal (LO) cortices, prelimbic cortex (PrL) and anterior cingulate cortex (Cg), nucleus accumbens core (AcbC) and shell (AcbSh), basolateral amygdala (BLA), substantia nigra pars compacta (SNc), ventral tegmental area (VTA), and dorsal (DRD) and ventral (DRV) subregions of the dorsal raphe nucleus. Sections are modified from the rat brain atlas of Paxinos and Watson (1997).

Acquired images were processed for several steps using ImageJ software. At first, brightness and contrast (B&C) of each image was adjusted automatically. Secondly, for each staining, a threshold value for immunopositive cells was determined manually using the auto local threshold option in ImageJ and the threshold for one staining was applied to all corresponding images. For cell counts, overlays of B&C-adjusted images and threshold images were created for NeuN-, D2- and 5-HT_{1A}-stainings and for the double-labeling of D2 and 5-HT_{1A} neurons. For analysis of receptor density, the percentage of immunopositive pixels from each threshold image was calculated relative to the total number of pixels in one image.

Examined brain areas were the ventral (VO, Bregma +4.6 to +3.8 mm) and lateral orbital (LO, Bregma +4.6 to +3.8 mm), the prelimbic (PrL, Bregma +4.0 to +3.2 mm) and anterior cingulate (Cg, Bregma +2.4 to +1.6 mm) cortices, the nucleus accumbens core (AcbC, Bregma +1.8 to +1.0 mm) and shell (AcbSh, Bregma +2.0 to +1.0 mm), the basolateral amygdala (BLA, Bregma -2.3 to -3.1 mm), the substantia nigra pars compacta (SNc, Bregma -5.0 to -5.6 mm) and ventral tegmental area (VTA, Bregma -5.3 to -6.0 mm) of the midbrain and the dorsal (DRD, Bregma -7.3 to -8.3 mm) and ventral (DRV, Bregma -7.3 to -8.3 mm) subregions of the dorsal raphe nucleus (DRN; Fig. 4.1).
For quantification, the size of each acquired image was identical with frame size which was 100.000 μm^2 (acquisition at 200x magnification) for frontal cortex regions, BLA, nucleus accumbens and midbrain structures and 41.500 μm^2 (acquisition at 400x magnification) for DRN subregions. Counting was performed manually and blind to behavioral group classifications and pixel numbers were determined automatically using ImageJ software.

4.3.5. Statistical analysis

Data were analyzed using IBM SPSS for Windows (version 26.0). In all analyses, a p value < 0.05 was considered statistically significant, and a p value between 0.05 and 0.1 was considered a non-significant trend.
For cell count data and percentage of immunopositive pixels, group comparisons were conducted separately for each brain region by parametric one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis test with behavioral group as between-subjects factor. All post hoc analyses were conducted using Bonferroni correction to detect detailed group differences. Group data were initially analyzed for normality using the Shapiro-Wilk test. Homogeneity of variance for each one-way ANOVA was verified using Levene's test. In case of violation of homogeneity, a Welch test with subsequent Games-Howell post hoc comparisons were used.
For analysis of D2 receptor density, data from one individual of the H-Imp group and one individual of the control group were excluded for the AcbC and AcbSh due to a high non-specific background staining.

4.4. Results

4.4.1. Behavioral data

Choice impulsivity in DA rats was assessed by use of a DD paradigm. For classification of the rats' impulsivity level, area under curve (AUC) values were used based on the preference for a large, delayed reward. Accordingly, four rats were classified as low impulsive (L-Imp, AUC > 0.67) and five individuals were classified as highly impulsive (H-Imp, AUC < 0.34). A detailed analysis of the behavioral profile of impulsive choice behavior in DA rats is provided in chapter 2 of the present dissertation. In the present experiment, individuals with lowest and highest values for choice impulsivity were chosen to investigate the highest possible differences between groups in terms of D2 and 5-HT_{1A} receptor expression.

4.4.2. Immunohistochemical data

To evaluate possible effects of behavioral measures on the number of D2- and 5-HT_{1A}-expressing neurons and the receptor density for both receptors, selected brain regions were investigated for NeuN-, D2- and 5-HT_{1A}-immunoreactivity in both impulsivity groups and a baseline control group. A representative photomicrograph from the anterior cingulate (Cg) cortex is shown in Fig. 4.2.

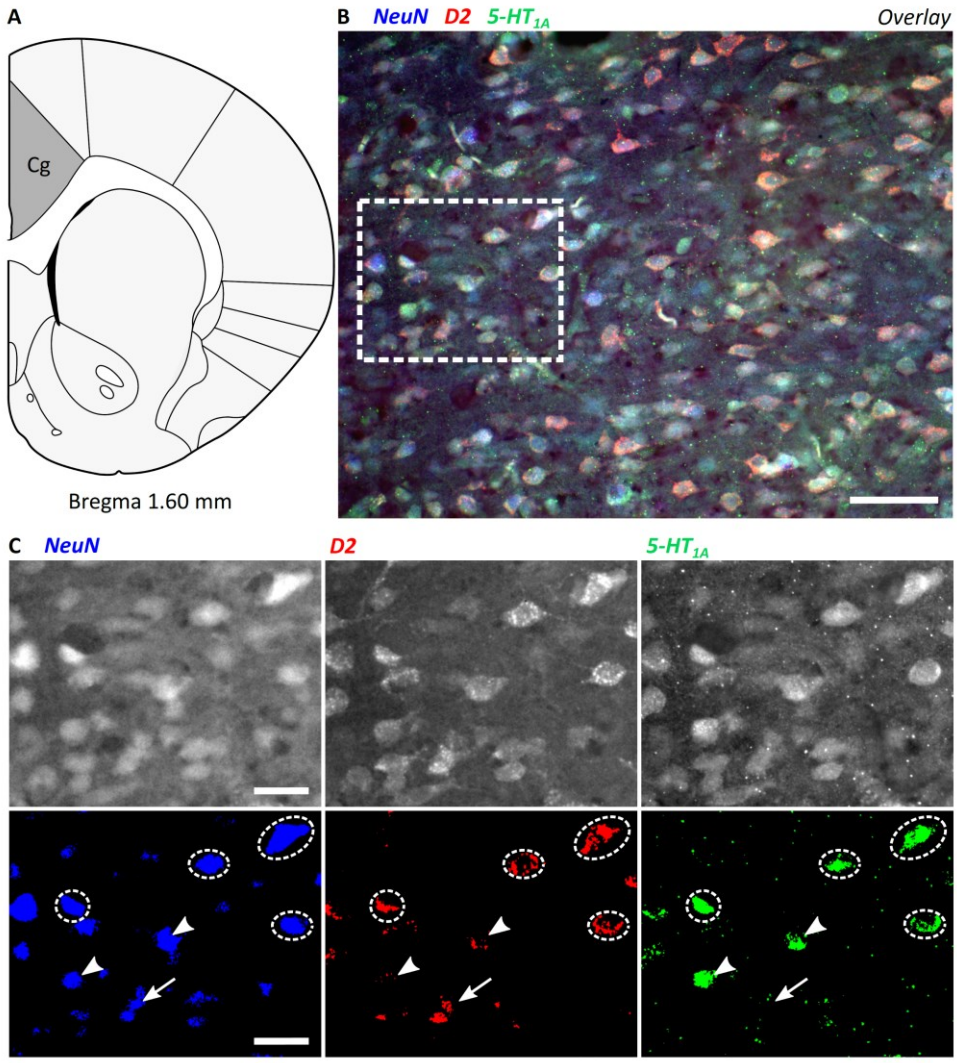

Figure 4.2 | Immunoreactivity of NeuN (blue), D2 (red) and 5-HT_{1A} (green) in the rat anterior cingulate cortex (Cg). A, Schematic drawing of a coronal section at the level of the striatum (Bregma 1.60 mm) from which images in B were captured (shaded region); adapted from Paxinos and Watson (1997). **B**, Representative photomicrograph of the immunohistochemical labeling of NeuN, D2 and 5-HT_{1A} shown as a three-channel overlay image. **C**, Single color channels are shown as enlarged selection from B (dashed rectangle) with grayscale images in the upper panel and corresponding threshold images with immunopositive labeling in the lower panel. Arrows, arrowheads and dashed circles illustrate neurons that express immunoreactivity for D2, 5-HT_{1A} and the combination of both, respectively. Scale bars are 50 µm for the overlay image in B and 20 µm for enlarged images in C.

4.4.2.1. Neuron numbers

VO. In the VO, statistical analysis revealed highly significant differences between behavioral groups for the NeuN staining ($F_{2,45}$ = 16.626, p < 0.001), while neuron numbers were constant between groups for D2 (H_2 = 2.744, p = 0.254), 5-HT_{1A} ($F_{2,45}$ = 0.839, p = 0.439), and double-immunopositive neurons ($F_{2,45}$ = 1.442, p = 0.247). Subsequent post hoc analysis showed a decreased number of NeuN-immunopositive neurons in L-Imp and H-Imp groups each compared to the control group (Fig. 4.3A).

LO. In the LO subregion, no significant differences between behavioral groups were observed (NeuN: $F_{2,44}$ = 0.632, p = 0.536; D2: $F_{2,44}$ = 0.313, p = 0.733; 5-HT_{1A}: $F_{2,44}$ = 2.165, p = 0.127; double: H_2 = 3.674, p = 0.159; Fig. 4.3).

PrL. Statistical analysis in the PrL revealed significant differences between behavioral groups for double-labeled neurons ($F_{2,45}$ = 3.320, p < 0.05). Post hoc analysis for double-labeled neurons, however, revealed no further group differences but a trend towards a decreased neuron number in H-Imp rats as compared to control rats (p = 0.083; Fig. 4.3D). No group effects were observed for NeuN ($F_{2,45}$ = 0.337, p = 0.716; Fig. 4.3A), D2 ($F_{2,45}$ = 0.619, p = 0.543; Fig. 4.3B) and 5-HT_{1A} ($F_{2,45}$ = 1.423, p = 0.252; Fig. 4.3C).

Cg. Overall significant differences between behavioral groups were found for 5-HT_{1A} ($F_{2,27.993}$ = 3.591, p < 0.05) and double-immunopositive neurons (H_2 = 6.825, p < 0.05) in the Cg, whereas no group effects were observed for NeuN ($F_{2,43}$ = 3.088, p = 0.056) and D2 (H_2 = 5.58, p = 0.061). Multiple comparisons showed a decreased 5-HT_{1A} count for L-Imp compared to H-Imp rats (Fig. 4.3C). For double-labeled neurons, post hoc analysis revealed non-significant trends towards a decreased neuron number in the L-Imp group compared to the H-Imp (p = 0.054) and the control group (p = 0.076) (Fig. 4.3D).

Thus, in all examined frontal cortex regions, the number of D2-expressing neurons was unaltered in both impulsivity groups. For 5-HT_{1A}, the only significant group difference was observed between L- and H-Imp rats in the Cg, while both impulsivity groups did not differ compared to the control. It is therefore not clear whether 5-HT_{1A} neuron numbers are decreased in L-Imp rats or increased in H-Imp rats compared to the other impulsivity group.

NAc. Statistical analysis in the NAc core region revealed significant differences between behavioral groups for NeuN ($F_{2,42}$ = 26.01, p < 0.001), D2 (H_2 = 16.974, p < 0.001) and the double-immunostaining ($F_{2,27.701}$ = 6.649, p < 0.01), whereas no difference was observed for 5-HT_{1A} ($F_{2,27.964}$ = 1.865, p = 0.174). In the NAc shell, cell counts differed for D2 ($F_{2,42}$ = 5.263, p < 0.01) and 5-HT_{1A} ($F_{2,25.756}$ = 7.908, p < 0.01), while neuron numbers were constant between groups for NeuN ($F_{2,42}$ = 0.212, p = 0.810) and double-labeling ($F_{2,42}$ = 0.712, p = 0.497). Subsequent multiple comparisons for the core region showed that NeuN, D2 and double-immunopositive counts in both impulsivity groups were decreased compared to the control (Fig. 4.3). However, differences in D2-expressing and double-labeled neurons are similar to differences in NeuN counts and were therefore excluded from further investigation. In the shell region, H-Imp rats had a decreased number of D2-labeled neurons compared to the control group (Fig. 4.3B). Post hoc analysis for 5-HT_{1A}-expressing neurons revealed an increased cell count for L-Imp rats and a trend towards an increase for H-Imp rats (p = 0.063) each compared to the control group (Fig. 4.3C). Hence, in both subregions of the NAc, impulsivity groups did not differ significantly from each other in all stainings. In the AcbC, the differences between impulsivity groups and control group for D2- and double-immunolabeling were found to correspond to differences in NeuN counts. In the AcbSh, however, D2-labeled neurons were decreased in H-Imp rats, whereas 5-HT_{1A}-labeled neurons were increased in L-Imp rats.

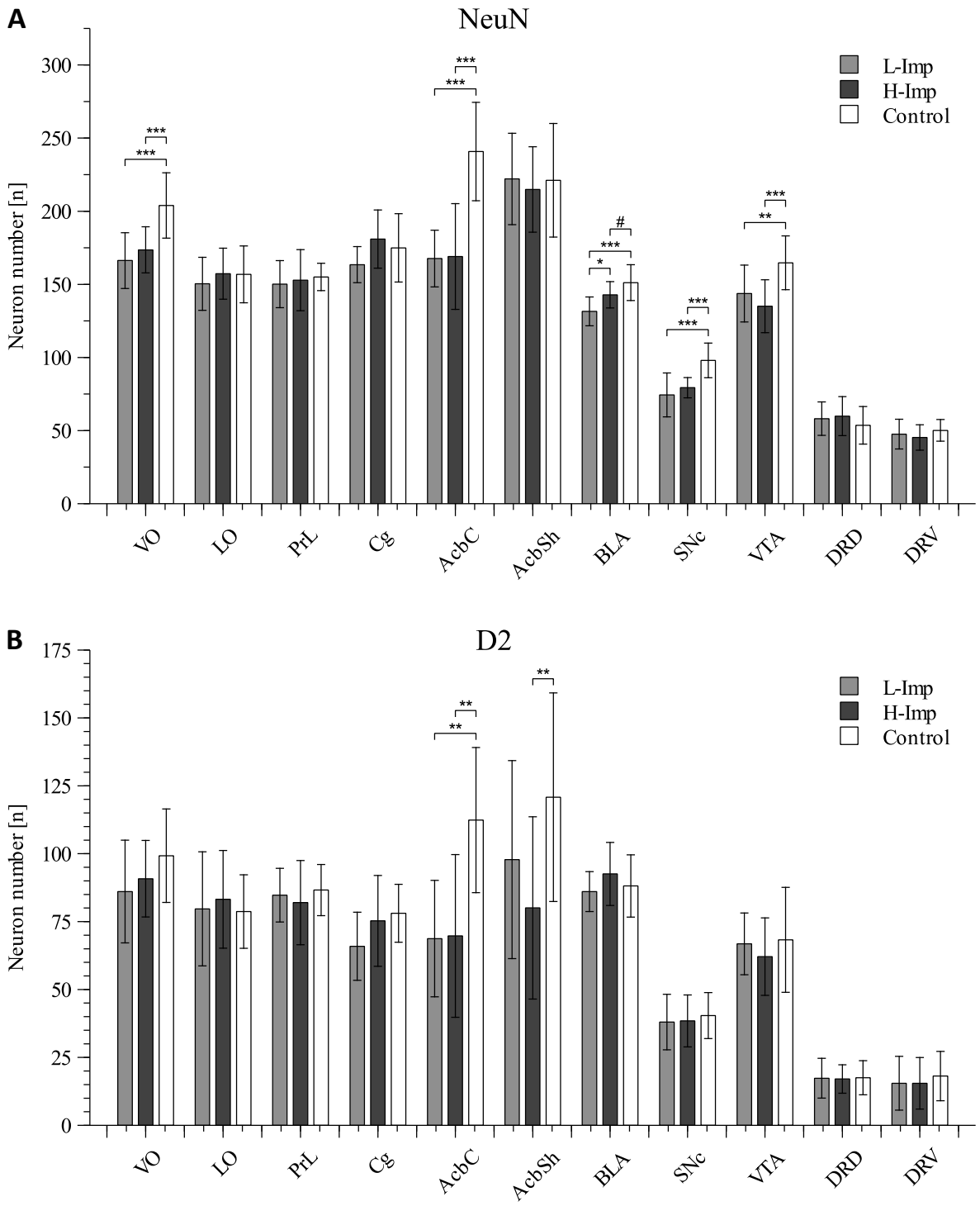

Figure 4.3 | Number of immunopositive neurons in selected brain regions of low-impulsive (L-Imp) rats, highly-impulsive (H-Imp) rats and a baseline control group. Cells were quantified for the expression of neuronal nuclei (NeuN, **A**), the D2 receptor (**B**), the 5-HT_{1A} receptor (**C**) and the double-labeling of both receptors (**D**). Data are expressed as mean ± SD. For abbreviations of brain regions see text. *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.1 (trend).

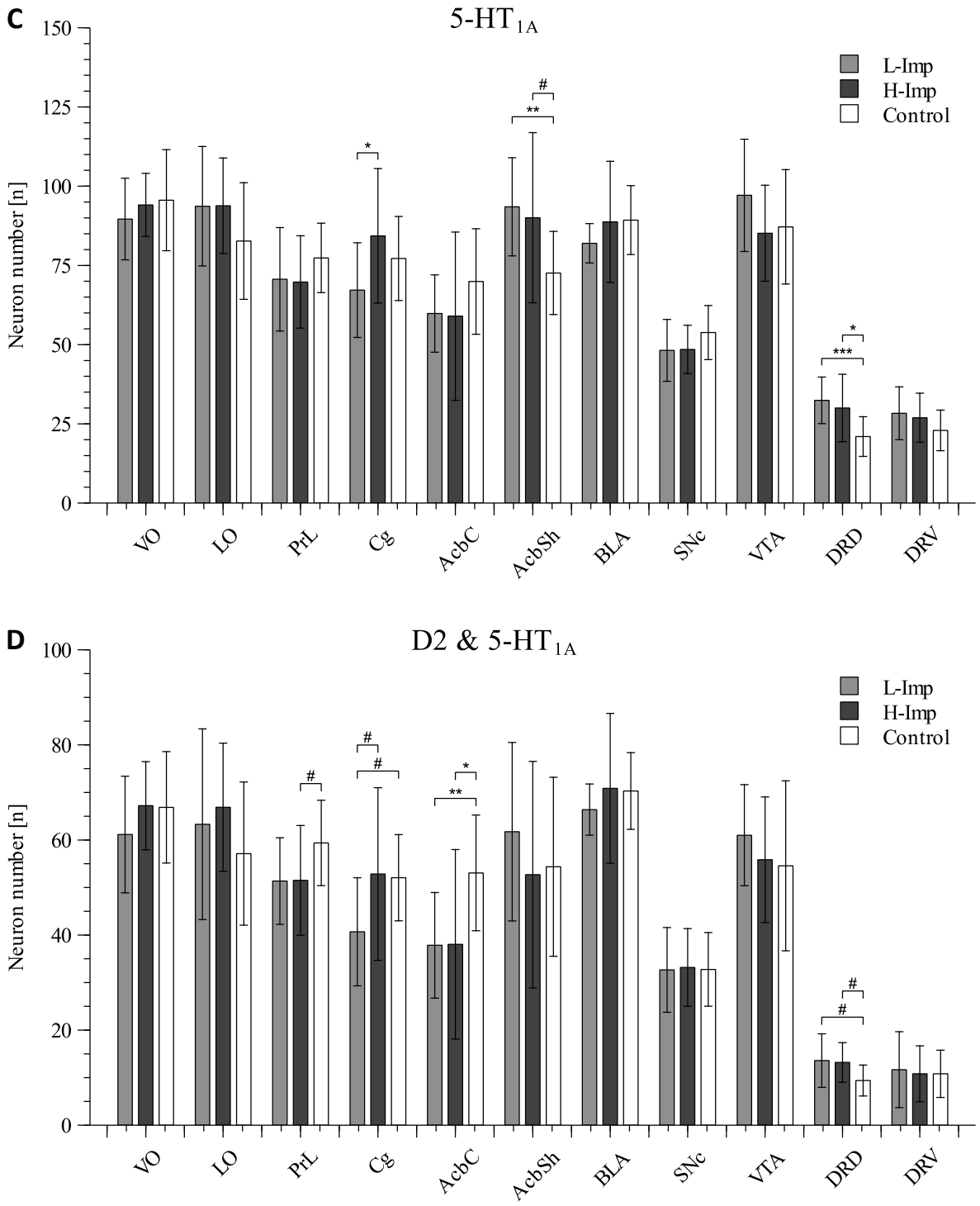

Figure 4.3 (Continued)

BLA. In the basolateral amygdala, overall significant group effects were found for NeuN ($F_{2,44} = 13.742$, $p < 0.001$), whereas no differences were observed for any other staining (D2: $F_{2,44} = 1.595$, $p = 0.214$; 5-HT_{1A}: $F_{2,26.012} = 3.08$, $p = 0.063$; double: $F_{2,26.735} = 1.546$, $p = 0.231$). Subsequent post hoc analysis revealed a decrease in NeuN-immunopositive cells for L-Imp rats as compared to H-Imp and control rats (Fig. 4.3A). Additionally, a trend towards a decrease in NeuN number was seen in the H-Imp group compared to the control ($p = 0.089$). Thus, no changes were found for both impulsivity groups within this region of the amygdala regarding D2- and 5-HT_{1A}-expressing neurons.

SNc. Statistical analysis in the SNc revealed highly significant differences between behavioral groups only for NeuN ($F_{2,26.510}$ = 18.077, p < 0.001), while neuron numbers were constant between groups for D2 ($F_{2,47}$ = 0.307, p = 0.737), 5-HT_{1A} ($F_{2,47}$ = 2.263, p = 0.115), and double-immunopositive neurons ($F_{2,47}$ = 0.017, p = 0.983). Further multiple comparisons showed a decreased number of NeuN-immunopositive neurons in L-Imp and H-Imp groups each compared to the control group (Fig. 4.3A).

VTA. In the VTA, overall significant group effects were observed for NeuN-immunopositive neurons ($F_{2,50}$ = 12.546, p < 0.001) and post hoc analysis showed a decrease for both impulsivity groups compared to the control (Fig. 4.3A). Statistical analysis for D2 ($F_{2,50}$ = 0.823, p = 0.445), 5-HT_{1A} ($F_{2,50}$ = 2.357, p = 0.105), and double-immunopositive neurons ($F_{2,50}$ = 0.903, p = 0.412) revealed no significant group effects. Hence, in the midbrain dopaminergic system, no differences were found between L-Imp and H-Imp rats or compared to control rats for the serotonin and the dopamine receptor subtypes.

DRN. In the dorsal raphe nucleus, NeuN numbers were constant between all groups for the dorsal and the ventral subregions (DRD: $F_{2,39}$ = 0.980, p = 0.384; DRV: H_2 = 2.624, p = 0.269). For the D2 staining, no differences between all groups were found as well (DRD: $F_{2,39}$ = 0.021, p = 0.979; DRV: $F_{2,39}$ = 0.378, p = 0.687). Regarding 5-HT_{1A}-immunopositive neurons, groups differed significantly in the dorsal subregion (H_2 = 14.184, p < 0.001) and post hoc analysis revealed a significant increase in both impulsivity groups compared to the control group (Fig. 4.3C). Within the ventral subregion, 5-HT_{1A} cell counts did not differ between groups ($F_{2,39}$ = 1.959, p = 0.155). Similar group effects as found for 5-HT_{1A} were seen for double-labeled neurons, such that groups differed significantly in the DRD (H_2 = 7.48, p < 0.05), while no difference was observed for the ventral subregion ($F_{2,23.509}$ = 0.058, p = 0.944). Subsequent multiple comparisons for the DRD, however, revealed no significant differences between groups, but trends towards increased neuron numbers for L-Imp (p = 0.051) and H-Imp (p = 0.064) rats as compared to the control group (Fig. 4.3D). Thus, in the DRV, no group effects were observed for all stainings. Additionally, in both examined subregions of the DRN, both impulsivity groups did not differ significantly from each other for all stainings. In the DRD, however, the number of 5-HT_{1A}-expressing neurons was significantly increased in both impulsivity groups.

4.4.2.2. D2 and 5-HT_{1A} receptor density

VO. Statistical analysis in the VO on the percentage of labeled pixels revealed significant differences between behavioral groups for the D2 receptor (H_2 = 7.424, p < 0.05) and subsequent multiple comparisons showed a decreased receptor density for H-Imp rats and a trend towards a corresponding decrease for L-Imp rats (p = 0.067) each compared to the control (Fig. 4.4A). For 5-HT_{1A}, no group differences were observed (H_2 = 1.384, p = 0.501).

LO. Statistical analysis revealed no significant differences between behavioral groups within the LO subregion for both receptors (D2: $F_{2,44}$ = 0.233, p = 0.793; 5-HT_{1A}: $F_{2,44}$ = 0.008, p = 0.993; Fig. 4.4A,B).

PrL. In the PrL, overall significant group effects were observed for D2 ($F_{2,45}$ = 4.667, p < 0.05) and 5-HT_{1A} ($F_{2,45}$ = 3.685, p < 0.05). Subsequent multiple comparisons revealed a decreased percentage of both receptors for H-Imp rats compared to the control (Fig. 4.4A,B). Additionally, a trend towards a decrease in D2 receptor density was observed for H-Imp rats compared to L-Imp rats (p = 0.057).

Cg. In the Cg, statistical analysis revealed significant differences between behavioral groups for 5-HT_{1A} (H_2 = 8.63, p < 0.05), whereas no group effects were observed for D2 (H_2 = 5.384, p = 0.068). Post hoc analysis showed a decreased 5-HT_{1A} receptor density for L-Imp compared to control rats and a trend towards a corresponding decrease compared to H-Imp rats (p = 0.077; Fig. 4.4B).

Hence, in the frontal cortical regions VO and PrL, D2 receptor density was decreased in H-Imp rats, whereas no differences were observed in LO and Cg. For L-Imp rats, no significant differences were found, although in the PrL a trend towards a decreased D2 receptor expression in the H-Imp group indicates a potential difference between both impulsivity groups.
For the 5-HT_{1A} receptor, no effects were found in the OFC subregions, while density was decreased for H-Imp rats in the PrL and for L-Imp rats in the Cg. Moreover, in the Cg, a non-significant trend indicated a potential difference between both impulsivity groups.

NAc. Overall significant group effects in the NAc core region were found for D2 ($F_{2,36}$ = 20.371, $p < 0.001$) and post hoc analysis showed a decrease in receptor density for both impulsivity groups compared to the control (Fig. 4.4A). For 5-HT_{1A}, percentage of labeled pixels did not differ between groups ($F_{2,27.806}$ = 1.361, p = 0.273). In the NAc shell, receptor density differed for the D2 ($F_{2,36}$ = 5.346, $p < 0.01$) and 5-HT_{1A} receptor ($F_{2,26.404}$ = 6.979, $p < 0.01$). Subsequent multiple comparisons for the shell region showed that D2 receptor density was decreased for H-Imp rats compared to the control (Fig. 4.4A), whereas 5-HT_{1A} receptor density was increased for the L-Imp group in comparison to the control group (Fig. 4.4B).

Thus, in both subregions of the NAc, both impulsivity groups did not differ significantly from each other for all stainings. However, in L-Imp and H-Imp groups, D2 receptor density was reduced by half compared to the control group in the core region. In the AcbSh, the D2 receptor was decreased in H-Imp rats and the 5-HT_{1A} receptor was increased in L-Imp rats. The latter results are similar to the effects observed for D2 and 5-HT_{1A} neuron numbers in this brain region.

BLA. In the basolateral amygdala, receptor density did not significantly differ between groups for both stainings (D2: $F_{2,44}$ = 0.913, p = 0.409; 5-HT_{1A}: H_2 = 3.728, p = 0.155; Fig. 4.4). Hence, impulsivity groups did not differ significantly with regard to D2 and 5-HT_{1A} receptor expression, which is consistent with D2 and 5-HT_{1A} neuron numbers in this brain region.

SNc. In the SNc, statistical analysis showed that receptor density was constant between groups for D2 ($F_{2,47}$ = 0.365, p = 0.696) and 5-HT_{1A} ($F_{2,47}$ = 0.494, p = 0.614) (Fig. 4.4).

VTA. In the VTA, highly significant group effects were observed for 5-HT_{1A} ($F_{2,50}$ = 9.332, $p < 0.001$) and post hoc analysis revealed that receptor density was increased in L-Imp rats compared to the control (Fig. 4.4B). Additionally, in H-Imp rats, a trend towards an increased 5-HT_{1A} density was observed compared to control rats (p = 0.069). No group effects were observed for D2 receptor density ($F_{2,50}$ = 1.83, p = 0.171). Hence, within the midbrain dopaminergic system, receptor density was only altered for the 5-HT_{1A} receptor in the VTA of L-Imp rats. Additionally, impulsivity groups were not significantly different from each other.

DRN. In the dorsal raphe nucleus, D2 receptor density was constant between all groups for the dorsal and the ventral subregions (DRD: $F_{2,39}$ = 0.506, p = 0.607; DRV: $F_{2,39}$ = 0.701, p = 0.502; Fig. 4.4A). For the 5-HT_{1A} staining, significant group differences were observed for both subregions (DRD: H_2 = 9.084, $p < 0.05$; DRV: $F_{2,39}$ = 4.977, $p < 0.05$). In the DRD and the DRV subregions, subsequent multiple comparisons revealed increased receptor density for both impulsivity groups compared to the control group (Fig. 4.4B). Thus, no changes were observed between impulsivity groups for all stainings and D2 receptor density was generally unaltered. The density of the 5-HT_{1A} receptor, however, was increased in both impulsivity groups in the DRD as well as the DRV.

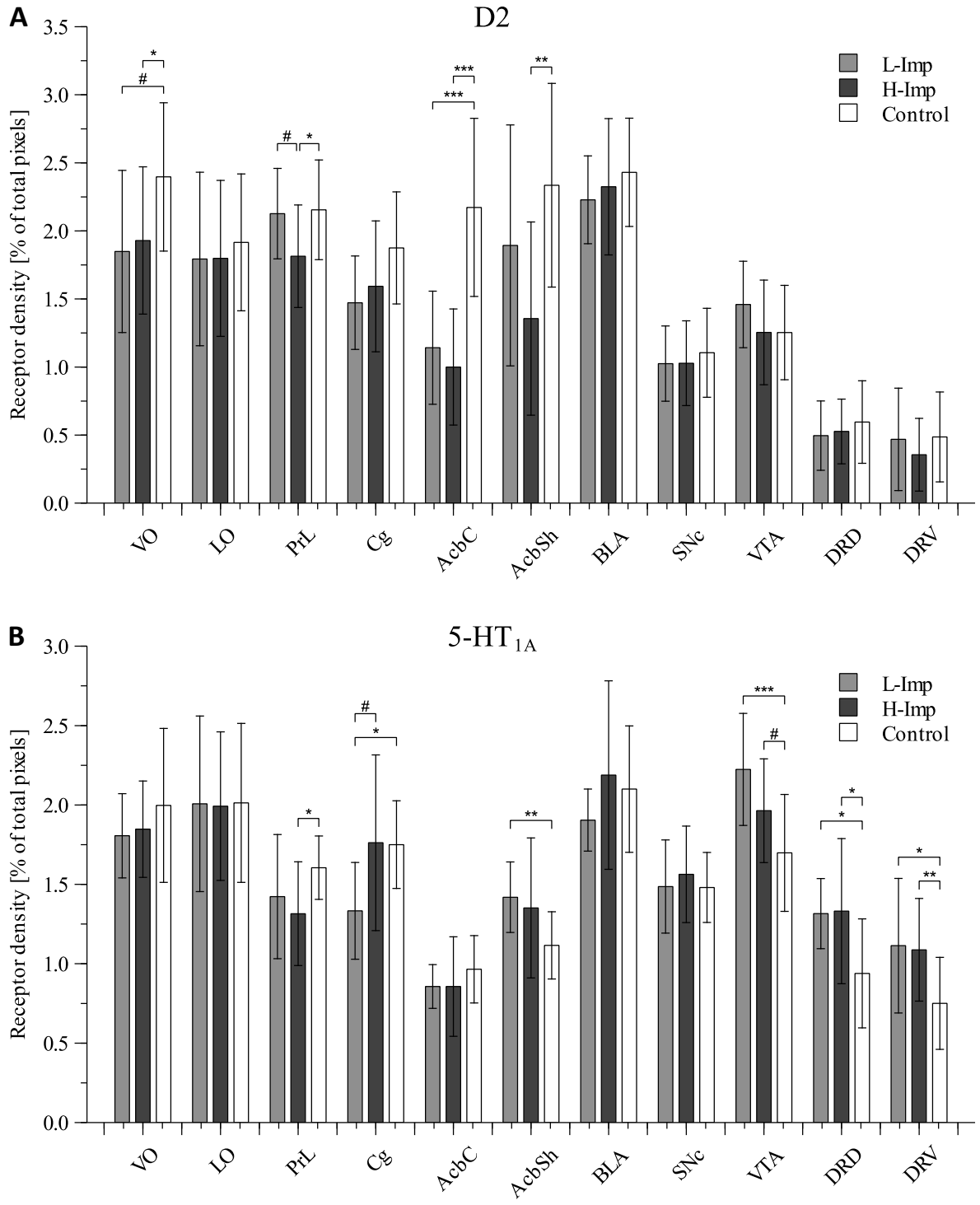

Figure 4.4 | Receptor density [% of total pixels] of D2 (A) and 5-HT$_{1A}$ (B) receptors in selected brain regions of low-impulsive (L-Imp) rats, highly-impulsive (H-Imp) rats and a baseline control group. Data are expressed as mean ± SD. For abbreviations of brain regions see text. *p < 0.05; **p < 0.01; ***p < 0.001; $^{\#}$p < 0.1 (trend).

4.5. Discussion

In the present study, rats that were classified into low- (L-Imp) and highly-impulsive (H-Imp) individuals in a DD task (see chapter 2) were further examined for the expression of the D2 and the 5-HT_{1A} receptor in selected brain regions relevant for this type of impulsive behavior.
The performance in a DD task overall affected the number of D2-expressing neurons in the AcbSh and D2 receptor density in VO, PrL, AcbC and AcbSh. For the 5-HT_{1A} receptor, neuron numbers were altered in Cg, AcbSh and DRD, while receptor density was affected in PrL, Cg, AcbSh, VTA, DRD and DRV.
Receptor expression in the LO as well as BLA and SNc were not affected by this delayed reward task.

4.5.1. D2 in impulsivity

Performance in the DD task resulted in changes in D2 receptor expression in cortical structures and in the nucleus accumbens of male Dark Agouti rats. Particularly, receptor density was decreased in VO and PrL subregions for H-Imp rats and in the AcbC for both impulsivity groups, without an effect on neuron number. In the AcbSh, both parameters were decreased, but only in H-Imp individuals. Hence, in these brain regions, D2 receptor expression was always altered in terms of a down-regulation of this receptor.
However, impulsivity-specific effects are only indicated in the PrL and the AcbSh, because significant differences from controls occurred for H-Imp rats, but not for L-Imp rats, and in the PrL an additional trend towards a difference between impulsivity groups was observed. This indicates an involvement of the D2 receptor in delay aversion in specific subregions of the frontal cortex and in the AcbSh.
The frontal cortex and the NAc are both components of the limbic corticostriatal loop and receive inputs from the midbrain dopaminergic system (for review see: Zhang, 2020). The D2 receptor is found in both of these structures where it functions as postsynaptic heteroreceptor and when activated reduces neuronal signaling of non-dopaminergic neurons (Beaulieu and Gainetdinov, 2011; Ford, 2014). The down-regulation of this receptor subtype observed in the present study might thus be associated with an increased neurotransmission of D2-expressing neurons in the frontal cortex and the NAc in rats that show an enhanced intolerance towards delays.
Previous pharmacological studies have also associated D2 receptor function in the frontal cortex with impulsive choice behavior. Administration of a D2-like receptor agonist was shown to increase choice impulsivity when injected into the mPFC, but not the OFC (Yates et al., 2014). However, the blockade of this receptor in the mPFC by D2-like antagonists was as well reported to increase choice behavior (Pardey et al., 2013; Yates et al., 2014). Findings for the OFC and D2 antagonists have shown both enhanced impulsive choice (Zeeb et al., 2010; Pardey et al., 2013) and no effect (Yates et al., 2014).
Due to the different methodical approaches, pharmacological findings are hard to compare with results from immunohistochemical investigations, but reduced D2 receptor expression as found in the present study and receptor blockade by antagonistic agents might have had similar stimulatory effects on neuronal signaling.
In the NAc, pharmacological manipulations of the D2 receptor were not found to affect choice impulsivity in rats (Yates and Bardo, 2017). Present findings are in contrast to those of Yates and Bardo, but since investigations of accumbal D2 receptor function are scarce, more research is needed to elucidate its specific role in impulsive choice behavior. Nevertheless, current results show an involvement of the shell region in delay aversion, whereas the AcbC appears to be involved in more general behavioral processes associated with delayed reinforcement (also see: J. S. Stein et al., 2013),

such as reward-related aspects of behavior, especially when considering the prominent functional role of the NAc in reward expectation (Martin and Ono, 2000; Winstanley et al., 2006a; de Boer et al., 2017). In contrast to local administration of D2 compounds, systemic administration of a D2-like agonist and different D2-like antagonists were reported to have no effect on choice impulsivity (Evenden and Ryan, 1996; van Gaalen et al., 2006; Koffarnus et al., 2011; Pattij et al., 2014; Li et al., 2015; Tian et al., 2019) with an exception for the antagonist raclopride which increased choice behavior (Wade et al., 2000). Given that D2 receptors are expressed as both presynaptic autoreceptors on dopaminergic neurons and postsynaptic heteroreceptors at dopaminergic projection sites (Beaulieu and Gainetdinov, 2011), systemic administration of D2 compounds in these studies potentially activated both receptor forms in various brain regions and thus may have counterbalanced the effects on impulsive choice behavior which emerged after local injections (also see: Yates et al., 2014).

4.5.2. 5-HT_{1A} in impulsivity

For 5-HT_{1A}, alterations in receptor expression were observed within cortical subregions, the NAc, as well as in the VTA and the DRN. Particularly, in PrL and Cg, receptor expression was decreased after DD performance, but with different results for impulsivity groups. While in the PrL receptor density was decreased in H-Imp individuals, this parameter was reduced in L-Imp rats in the Cg subregion.
Moreover, among all investigated structures, the Cg was the only area for which impulsivity groups differed significantly from each other in terms of 5-HT_{1A} neuron numbers. The direction of this effect, however, was inconclusive, because no differences were found relative to control. Given that receptor density was decreased for L-Imp rats compared to control and that a trend towards a group difference between L- and H-Imp groups was found, it is most likely that 5-HT_{1A} receptor expression within the Cg was overall reduced in L-Imp individuals rather than enhanced in H-Imp rats. Consequently, the changes observed for 5-HT_{1A} receptor expression in the mPFC were always in the form of a down-regulation and in the Cg subregion these effects appear to be specific for rats that tolerate longer delays.
In contrast, in AcbSh, VTA and the DRN, the 5-HT_{1A} receptor was affected by an up-regulation, because receptor density was increased in all of these structures, and in the AcbSh and the DRD, neuron numbers were increased as well. However, impulsivity groups did not differ significantly from each other. Additionally, in DRN subregions, both impulsivity groups showed significant changes compared to control rats. In AcbSh and VTA significant increases were only observed for L-Imp rats, but neuron numbers in the AcbSh and receptor density in the VTA of H-Imp individuals showed a trend towards an increase compared to the control group, indicating similar expression levels in both impulsivity groups. Therefore, alterations in the latter structures are presumably independent of impulsivity level.
The 5-HT_{1A} receptor is known to exert inhibitory effects on neuronal signaling (Barnes and Sharp, 1999; Millan et al., 2008). In the present study, an increase in receptor expression might thus indicate an enhanced inhibition of 5-HT_{1A}-expressing neurons, while a corresponding decrease might result in stimulatory effects on neurotransmission.
In rodents, the 5-HT_{1A} receptor was shown to be located within various brain regions, including dorsal and median raphe nuclei, in which the neurotransmitter serotonin is mainly synthesized (Grigoryan, 2012; Nakamura, 2013). Among all serotonergic projection areas, the frontal cortex, septum, striatum, hippocampus, amygdala and hypothalamus were reported to express high levels of the 5-HT_{1A} receptor (Barnes and Sharp, 1999; Aznar et al., 2003; Santana et al., 2004; Millan et al., 2008). In these areas, 5-HT_{1A} mainly functions as heteroreceptor and regulates neuronal signaling at the postsynapse,

whereas in the DRN, it is expressed as presynaptic autoreceptor, where it suppresses serotonin synthesis and release due to negative feedback mechanisms (Mengod et al., 2015).
Early pharmacological studies examined the effects of central serotonin depletion on choice impulsivity and often reported an increase in the preference for immediate versus delayed rewards (Wogar et al., 1993; Bizot et al., 1999; Mobini et al., 2000). In these studies, the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) was used to inhibit serotonin synthesis within the raphe nuclei which was also shown to effectively diminish serotonin levels within cortical projection areas (Winstanley et al., 2003). Hence, an increase in receptor expression as observed in the present study might be associated with enhanced inhibition of the DRN and diminished serotonin release following performance of a DD task. However, Winstanley and colleagues did not find an influence on choice behavior by use of 5,7-DHT (Winstanley et al., 2003, 2004a).
Furthermore, involvement of the 5-HT_{1A} receptor in choice impulsivity was previously investigated using selective 5-HT_{1A} agonists or antagonists. Hereby, systemically administered 5-HT_{1A} agonists were shown to either increase (Evenden and Ryan, 1999; Winstanley et al., 2005; van den Bergh et al., 2006a; Stanis et al., 2008; Blasio et al., 2012) or decrease choice impulsivity (Bizot et al., 1999; Zaichenko et al., 2013). A decrease was also reported after local injection of the agonist 8-OH-DPAT into the OFC, but not the mPFC (Yates et al., 2014). Antagonism by WAY 100635, on the contrary, was reported to have no effect on choice behavior, neither after systemic administration (Evenden and Ryan, 1999; Winstanley et al., 2005; Zaichenko et al., 2013), nor after local infusions into mPFC or OFC (Yates et al., 2014).
Accordingly, findings on 5-HT_{1A} involvement in choice behavior by use of pharmacological methods are mixed, which may be explained by a dual effect of selective compounds resulting in simultaneous activation of hetero- and autoreceptors, as previously described (de Boer and Koolhaas, 2005; van den Bergh et al., 2006a).
In the present study, changes in 5-HT_{1A} receptor expression, especially its up-regulation, were associated with the performance of the DD task but were not explicitly found to relate to different levels of impulsivity. One explanation may be that this receptor subtype is involved in reward-mediated processes relevant for DD task performance rather than temporal processes that are required for delay-based decisions. This assumption is supported by previous findings for the DRN that different subsets of neurons are activated by the prediction and receipt of either large or small rewards (Nakamura, 2013). Similar changes in 5-HT_{1A} receptor expression might therefore arise in individuals with varying behavioral levels as seen in the present study with L- and H-Imp rats.

4.5.3. Brain areas in choice impulsivity

The brain areas examined in the present study have all previously been linked to impulsive choice behavior, often investigated by lesion studies (for review see: Baunez and Lardeux, 2011; Cardinal, 2006; Dalley et al., 2011). However, investigations of receptor-specific effects, including D2 and 5-HT_{1A}, are less abundant for specific brain regions (see above). Present results associate the performance in delayed reinforcement as assessed on a DD task with D2 receptor function in OFC, mPFC and NAc, and with 5-HT_{1A} receptor function in the mPFC, NAc, VTA and DRN. The frontal cortex, NAc and VTA are highly interconnected brain regions which constitute components of the limbic corticostriatal loop and regulate reward-related aspects of behavior (Winstanley, 2007; Roesch and Bryden, 2011). Current results suggest that both receptor subtypes operate in overlapping neuronal networks that are relevant for DD-task performance. In this regard, one interesting finding of the present study is that 5-HT_{1A} was affected in the DRN and thus the main area of serotonin synthesis, whereas a corresponding

effect for the D2 receptor and the VTA was not observed, although both receptor subtypes are known to act as presynaptic autoreceptors on serotonergic and dopaminergic neurons, respectively (Ford, 2014; Mengod et al., 2015; Gallo, 2019). Instead, 5-HT_{1A} receptor expression was enhanced in the VTA, and D2 receptor expression was confined to dopaminergic projection sites.
The BLA, for which no changes in receptor expressions were found in the present study, is another component of the aforementioned reward circuitry and its inactivation has been previously reported to increase choice behavior (Winstanley et al., 2004b; Churchwell et al., 2009). The BLA was found to play an important role in reward processes that are linked to learning and memory (Winstanley et al., 2004b, 2006a; Nakamura, 2013). Although both receptors were generally detected within the BLA, the present study does not provide evidence for an involvement of this brain region in choice impulsivity. Present results are consistent with a study by Meda et al. (2019) which investigated the relations between choice behavior and several dopamine receptors in different neural pathways projecting from cortical regions to the BLA. For the D2 receptor, the authors found no correlations with choice impulsivity in neither of these pathways. Thus, the BLA might be involved in inter-temporal choice via different serotonergic or dopaminergic receptors than those examined in the present study.
In the SNc, D2 and 5-HT_{1A} receptors were likewise not affected by the DD task, which is consistent with an earlier lesion study which reported no effect on choice impulsivity as well (Magnard et al., 2018). Although the SNc is another source of dopamine, it appears to play a less prominent role in DD performance, while its projection to the dorsal striatum might be of more importance, because lesioning of this region was shown to increase impulsive choice (Tedford et al., 2015).

4.5.4. Interactions between dopamine and serotonin

Given that receptor expression was altered for both D2 and 5-HT_{1A} in the PrL and in the AcbSh, these areas may play a role in potential receptor interactions in choice impulsivity or the execution of a DD task. In particular, while in the PrL both receptors were down-regulated in H-Imp rats, a down- and an up-regulation were found in the AcbSh for D2 and 5-HT_{1A}, respectively. Hence, one might speculate that interactive effects of these receptors on behavioral outcome might be differentially expressed in each of these brain regions.
Previous studies already provided evidence for an interaction between the dopaminergic and the serotonergic system in the regulation of impulsive choice behavior (Winstanley et al., 2005, 2006b). For example, in a microdialysis study by Winstanley and colleagues, who examined the specific role of serotonin and dopamine neurotransmission induced by choice impulsivity, behavioral performance enhanced serotonin and dopamine efflux in the mPFC, whereas in the OFC, including VO and LO, only dopamine was affected (Winstanley et al., 2006b). Present results are consistent with these earlier findings, insofar as expression levels of both receptors were affected in the mPFC, whereas in the OFC only D2 receptor expression was altered. Winstanley and colleagues additionally investigated 5-HT-DA-interactions on choice behavior by pharmacological manipulations of both transmitter systems within the NAc (Winstanley et al., 2005). The authors reported that activation of the 5-HT_{1A} receptor by the selective agonist 8-OH-DPAT increased choice impulsivity, while selective dopamine depletion by the neurotoxin 6-hydroxydopamine (6-OHDA) had no effect. The combination of both substances, however, blocked the effect of 8-OH-DPAT in increasing choice behavior. Therefore, Winstanley and colleagues suggested an influence of the 5-HT_{1A} receptor on dopaminergic neurotransmission in the NAc, although the exact mechanisms of these interactions remain to be elucidated. Moreover, findings from the latter study and the present experiment both demonstrate an association of serotonergic and dopaminergic systems in the NAc with delayed reinforcement.

4.5.5. Conclusion

Taken together, results from the present study show differential alterations of dopamine D2 and serotonin 5-HT_{1A} receptor expression in response to a delayed-reward task in male Dark Agouti rats. While behavioral performance led to reduced expression levels of the D2 receptor in VO, PrL and both accumbal subregions, changes in 5-HT_{1A} receptor expression were subjected to a down-regulation in the mPFC (Cg) and to an up-regulation within AcbSh, VTA and DRN.

However, specific changes associated with impulsivity level were only present within the mPFC for both receptors and within the NAc for D2, such that D2 expression was decreased in H-Imp individuals and 5-HT_{1A} expression was decreased in L-Imp rats.

Thus, present results indicate that an enhanced intolerance towards delays is associated with diminished D2 receptor function in dopaminergic projection areas. Moreover, this suggests a primary role for D2 heteroreceptors in DD task performance, because no effects were observed in VTA and SNc, i.e., the midbrain dopaminergic system where D2 acts as presynaptic autoreceptor. Given the inhibitory effect of D2 receptors, diminished heteroreceptor function in frontal cortex and NAc might have promoted neuronal signaling in rats that show increased delay aversion.

For the 5-HT_{1A} receptor, the Cg appears to play a crucial role in rats with an enhanced ability to tolerate delayed rewards. As described for the D2 receptor, diminished 5-HT_{1A} heteroreceptor function presumably stimulated neuronal signaling within this frontal cortex subregion. Present findings additionally show that 5-HT_{1A} was increased in AcbSh, VTA and DRN, indicating inhibition of neuronal signaling in these regions. Given that these effects were independent of impulsivity level, 5-HT_{1A} receptor function in these areas appears to be involved in general behavioral processes associated with delayed reinforcement. However, it may alternatively be involved in the processing of reward-related aspects of behavior rather than temporal mechanisms required for delay-based decisions, potentially resulting in similar effects on 5-HT_{1A} receptor expression in individuals with varying levels of choice impulsivity.

Chapter 5

General discussion

Impulsivity and aggression are of considerable interest for neurobiological and psychiatric research because they represent aspects of behavior which occur naturally in response to environmental challenges or threats. When expressed excessively, impulsivity and aggression can contribute to personality problems or neuropsychiatric disorders. Studies in humans and rodents have made substantial progress in identifying the complex mechanisms and structures underlying these behaviors (Natarajan and Caramaschi, 2010; Winstanley, 2011; Takahashi and Miczek, 2014; Robbins and Dalley, 2017). However, there are still many open questions concerning the relationship of impulsivity and aggression and the form of impulsivity that predominantly contributes to impulsive-aggressive phenotypes in healthy individuals. The present thesis aimed at investigating this link between choice impulsivity and territorial aggression in male Dark Agouti (DA) rats. At the behavioral level, the current data demonstrate that both behaviors are expressed as a trait characteristic in this rat strain, but a causal link between both traits was not observed. The present work further revealed that the neuronal networks underlying impulsivity for the most part are distinguished from those regulating aggressive behavior. The overlapping brain areas identified in the current thesis included the frontal cortex and the dorsal raphe nucleus (DRN) with a particular involvement of the orbitofrontal cortex (OFC) in delay aversion as well as diminished aggressive behavior. Moreover, the medial prefrontal cortex (mPFC) was crucially implicated in impulse-control mechanisms mediated via the monoaminergic receptors 5-HT_{1A} and D2. In contrast to these frontal cortical areas, the subcortical structures nucleus accumbens (NAc) and basolateral amygdala (BLA) appear to contribute to the control of aggression rather than impulsive choices, although in both accumbal subregions the 5-HT_{1A} and D2 receptors were also affected by delay-discounting (DD) performance.

5.1. Behavioral aspects of impulsivity and aggression in DA rats

The findings described in chapter 2 demonstrate that male DA rats display strong individual differences in impulsive choice behavior, ranging from low to high levels, which means that several individuals showed high self-control**,** while others displayed an enhanced aversion towards delays. Given that this behavior stabilized over the course of the DD task, it is regarded as a trait characteristic in these rats. A large spectrum of the impulsivity trait is most often observed in outbred than inbred rat strains, which is why the DA strain demonstrates an exception to the previously reported behavioral profiles and it may be a suitable model strain for the analysis of different behavioral manifestations in choice impulsivity.

Offensive territorial aggression in DA rats, as assessed by a series of resident-intruder (RI) tests, on the contrary, was confined to low and medium levels. The narrow range of aggression of the current cohort of rats therefore differs from previous research from our laboratory which identified highly-aggressive (H-Agg) individuals and thus reported the full spectrum of aggression for the DA strain (Radant, 2010). A follow-up study from our laboratory has shown that H-Agg behavior can occur in DA rats, but was suggested a state characteristic in this strain (Arlt, 2013). While trait aggression reflects a stable personality characteristic, the actual expression of aggressive acts is described as state aggression (de Boer et al., 2003; van der Vegt et al., 2003). It was shown that the duration of offensive aggression often varied between single RI tests for individual rats, especially in rats which displayed

high aggression levels (Arlt, 2013). The classification of high aggression was based on a duration of offensive behavior higher than 55 % in a RI test (de Boer et al., 2003).
More particularly, in the study by Arlt (2013), only one individual had a constant assignment to one aggression level, while this was observed for the majority of rats of the present work (57% of rats; individual data not shown). Moreover, in the current cohort, one individual displayed H-Agg behavior in a single RI test (data not shown), whereas high aggression was more often observed in the study by Arlt (2013). Arlt therefore suggested that the individual fluctuations in offensive aggression and specifically the occurrence of H-Agg behavior represents state aggression rather than aggression as a trait.
Collectively, previous and present findings indicate that high levels of offensive aggression in the DA strain vary between distinct generations or populations of DA rats. In this regard, it might be argued that the low to intermediate range of aggression reflects a trait characteristic in DA rats, while the irregularly expressed H-Agg levels might represent state aggression instead (also see de Boer et al., 2017).
Regarding the relationship between both examined behaviors, the results described in chapter 2 demonstrate that choice impulsivity and territorial aggression do not correlate with one another. This finding contrasts with earlier reports in human subjects (Coccaro et al., 2011) and rodents under laboratory conditions (van den Bergh et al., 2006b; Cervantes and Delville, 2007; Rudebeck et al., 2007) that increased impulsivity is associated with increased aggression, and vice versa.
One possible explanation is that both traits do not correlate due to the different ranges within each behavioral spectrum, also in view of the absence of a H-Agg phenotype. In other words, the dimensions of the impulsivity spectrum do not coincide with those of the aggression spectrum. For example, we identified individuals showing low impulsivity together with little aggression, and low impulsivity together with medium levels of aggression. Hence, several different combinations of impulsive-aggressive phenotypes occurred in the DA strain.
Furthermore, choice impulsivity might not be required or only marginally relevant for the execution of aggressive acts displayed in RI confrontations. During the invasion of a territory, the resident needs to decide how to appropriately react to its opponent. Within the framework of an adaptive aggression range, such a reaction commonly includes threatening behavior followed by offensive attacks or the choice for tolerating the opponent due to withdrawal behavior displayed in response (Koolhaas et al., 2013; Takahashi and Miczek, 2014). I initially assumed that impulsive choice behavior might be relevant for the decision-making process of choosing among these options. In this context, an increased number of offensive attacks or an excessive duration of aggressive behavior might be the consequence of poor behavioral choices (see also: Blair, 2016; Van den Bergh et al., 2006) resulting in negative consequences, such as high energy consumption or an increased risk of injuries (see also: Haller, 2017). However, the results of chapter 2 do not confirm the initial assumption and rather point to independent behavioral processes for choice impulsivity and territorial aggression in male DA rats.

5.2. Impulsivity and aggression: Is there a common neuroanatomical basis?

The finding of chapter 2 that impulsive choice and territorial aggression in DA rats are not linked at the behavioral level fits well with data from immunohistochemical investigations of neuronal activation patterns for both behaviors in chapter 3 of the present work. Overall, the combined results from both studies demonstrate that impulsivity and aggression have a distinct neuroanatomical basis in the DA strain, because the examined brain regions were differentially activated by choice impulsivity in the DD task and aggressive acts in inter-male confrontations.

The current paragraph will focus on impulsivity and aggression with regard to neuronal activation patterns to illustrate the differences and similarities in the neuroanatomical basis of these two behaviors. The differences in neuronal networks between low- (L-Imp) and highly-impulsive (H-Imp), as well as low- (L-Agg) and medium-aggressive (M-Agg) groups will be discussed in sections 5.3. and 5.4., respectively. Results for chapter 4 which examined monoaminergic receptors in both impulsivity groups are included in section 5.3.

Altogether, figures 5.1. and 5.2. summarize the results for c-Fos expression levels of chapter 3 and changes in monoaminergic receptor expression of chapter 4 and they give an overview of all examined brain regions of the present work. Particularly, the results described in chapter 3 show that following the DD task, neuronal activation levels were changed in the VO and the DRN. The confrontation with an intruder, on the contrary, induced changes in several frontal cortex regions, the entire NAc and the BLA. Thus, the only overlap between impulsivity and aggression was observed in the VO subregion of the frontal cortex.

The observed effects on neuronal activation in the VO were decreased c-Fos expression levels following both tasks. For impulsivity, this demonstrates that the inhibition of the VO is specifically associated with an increased intolerance towards delays. In parallel, its inhibition appears to be necessary to suppress aggressive behavior. Accordingly, the decreased activity of this orbitofrontal subregion is linked to highly-impulsive, but low-aggressive behavior in DA rats.

Previous research has also shown that impulsive choice and aggressive behavior are each linked to OFC function (Baunez and Lardeux, 2011; Cardinal, 2006; Grigoryan, 2012; Halász et al., 2006; Miczek et al., 2015). In choice impulsivity, the OFC is associated with reward value integration (Mobini et al., 2002; Cardinal, 2006; Winstanley et al., 2006b; Fitoussi et al., 2015), value representation during task performance (Schoenbaum et al., 2011; Rudebeck and Murray, 2014) and evaluation of reward outcome (Wallis, 2012). With regard to aggressive behavior, the OFC might as well play a role in the evaluation of expected outcomes in territorial confrontations with conspecifics to provide a basis for appropriate responses (also see: Blair, 2016; Izquierdo et al., 2005; Rudebeck et al., 2007). Thus, present results confirm previous findings in that the VO subregion is implicated in the regulatory control of impulsive choice behavior and territorial aggression.

At the same time, our findings support the involvement of the OFC in choice impulsivity as opposed to the mPFC. This is consistent with previous literature which reported OFC involvement in delayed reinforcement by c-Fos induction (da Costa Araújo et al., 2010) as well as OFC lesions (Mobini et al., 2002; Winstanley et al., 2004b; Rudebeck et al., 2006; Mar et al., 2011), although the direction of lesion effects was often inconsistent. Some studies even observed no effect following OFC inactivation (Mariano et al., 2009; Abela and Chudasama, 2013). However, for the mPFC, no effects were reported on neuronal activation levels (da Costa Araújo et al., 2010) or after mPFC lesions (Cardinal et al., 2001; Rudebeck et al., 2006). Therefore, several lines of evidence show that the OFC plays an important role in impulsive choice behavior. This topic will be further discussed in section 5.3. with regard to monoaminergic receptors.

A different picture emerges for territorial aggression and the frontal cortex regions. Present and previous findings observed changes in neuronal activation in both the OFC and the mPFC induced by inter-male confrontations (Toth et al., 2012; Biro et al., 2017). This is in line with OFC and mPFC lesions which both increased aggressive behavior in rats (Rudebeck et al., 2007; Takahashi et al., 2014). Hence, the OFC and the mPFC are both heavily implicated in the regulation of aggressive behavior in the context of territorial intrusion.

In subcortical structures, i.e., the NAc and the BLA, neuronal activation was not affected by delayed reinforcement in DA rats, but after RI confrontations, the AcbSh was inhibited in M-Agg rats and the

BLA was inhibited in L-Agg individuals. Hence, regarding inter-male confrontations, the inhibition of AcbSh neurons appears to promote the expression of offensive aggression, while the inhibition of the BLA indicates the opposite effect, namely the suppression of aggressive behavior. Nevertheless, no interaction was found between choice impulsivity and territorial aggression in these brain regions.

Earlier studies, however, have implicated the NAc and the BLA in both behaviors. On the one hand, this was frequently investigated by lesion studies and, on the other hand, by c-Fos induction. In delayed reinforcement, it was reported that lesions of the NAc and the BLA resulted in increased choice impulsivity (Cardinal et al., 2001; Winstanley et al., 2004b; Pothuizen et al., 2005; Bezzina et al., 2007; Churchwell et al., 2009; da Costa Araújo et al., 2009; Galtress and Kirkpatrick, 2010; Valencia-Torres et al., 2012; Feja et al., 2014), which is contradictory to present findings. In line with NAc lesions, da Costa Araújo et al. (2010) have found an enhanced c-Fos expression in the NAc after performance of an adjusting-delay procedure. The latter approach separately evaluates delay sensitivity, which is considered one major element underlying impulsive choice; another element regards the sensitivity to reward sizes (Dalley et al., 2004; Marshall et al., 2014; Saddoris et al., 2015; Smith et al., 2015; Marshall and Kirkpatrick, 2016). A DD task, as used in the present work, implies alterations in both delay length and reward magnitude and it allows the assessment of impulsive choice behavior per se. Although impulsive behavior can be independently influenced by delay and reward mechanisms (Ho et al., 1999; Pitts and Febbo, 2004), a significant effect in one of these parameters does not inevitably imply changes in choice behavior as well. Accordingly, the absence of an effect as observed for c-Fos levels in the NAc of the present work and a significant effect in delay sensitivity reported by da Costa Araújo and colleagues (2010) do not necessarily exclude one another.

The NAc has been strongly implicated in reward-related behavior, especially reward expectation (Martin and Ono, 2000), and it is regarded as a key structure in the reward circuitry with connections to the frontal cortex, hippocampus, hypothalamus, amygdala and VTA (for review see: de Boer et al., 2017; Hu, 2016; Zhang, 2020). Accordingly, I initially anticipated changes in neuronal activation in the NAc in response to a DD task. In parallel, I assumed a corresponding effect for the BLA due to its reported functional implications in reward-related learning (Winstanley et al., 2004b, 2006a) and the assessment of reward value (Fitoussi et al., 2015).

However, the results described in chapter 3 do not support an involvement of the NAc and the BLA in delayed reinforcement. Nevertheless, according to the current literature, the BLA has not been investigated for c-Fos expression levels with regard to impulsive choice. Hence, this is the first study to address this issue.

The involvement of the NAc and the BLA differs for territorial aggression, because of the observed inhibitory effects in both of these brain regions following inter-male confrontations. In addition, chapter 3 revealed that impulsivity and aggression groups significantly differed from each other in the NAc and the BLA, which strengthens the conclusion that the subcortical structures investigated in the current work differentially contribute to trait impulsivity and trait aggression.

In addition to reward-related functions, the BLA is implicated in the evaluation of social behavior and fear responses (Duvarci and Pare, 2014; Felix-Ortiz and Tye, 2014), which are relevant aspects for intra-specific communication during RI confrontations. In earlier c-Fos induction studies, increases in neuronal activation in the BLA were associated with increased aggressive behavior (Halász et al., 2002a; Veening et al., 2005; Veenema et al., 2007; Toth et al., 2012; Konoshenko et al., 2013; Hong et al., 2014; Biro et al., 2017). Present results are in line with such a positive link between BLA involvement and aggression, but with the opposite direction of the effect. In male DA rats, BLA inhibition was associated with diminished aggression in contrast to BLA activation and increased aggression in the

literature. Thus, present findings point towards a role of the BLA in keeping aggression levels low or potentially maintaining aggression in an adaptive range.

A fight-induced increase in c-Fos levels was also reported earlier for the NAc, more specifically for the AcbSh subregion (Toth et al., 2012), implicating this brain area in the display of aggression. In chapter 3 of the present work, on the contrary, the AcbSh was inhibited in individuals that displayed aggressive behaviors. The direction of effects is thus opposed to earlier findings, similar to observations for the BLA. Nevertheless, changes in c-Fos expression in the AcbSh of DA rats did not differ between L- and M-Agg groups and were thus not specific for aggression level. The additional finding that c-Fos levels were significantly decreased in both aggression groups as compared to both impulsivity groups indicates that the confrontation with a conspecific itself induced an inhibition of both NAc subregions (data not shown in Fig. 5.2). Given its central role in reward-related behavior, the NAc may thus be involved in reward mechanisms related to social confrontations, irrespective of the execution of aggressive acts.

From the literature, one behavioral aspect potentially linking the accumbens region with territoriality is aggression-associated reward (Beiderbeck et al., 2012; Aleyasin et al., 2018b; Golden et al., 2019). In particular, repeated winning experience was reported to have a positive reinforcing effect which encourages dominant residents to engage in aggressive acts in subsequent inter-male encounters (Hsu et al., 2006; de Boer et al., 2009; Kerman et al., 2011; Falkner et al., 2016). In addition, operant conditioning procedures which use the access to an opponent as a reward have shown that mice with prior winning experience are eager to work for this type of reinforcement (Couppis and Kennedy, 2008) (Golden et al., 2019).

The involvement of the NAc in such rewarding effects and motivational processes linked to aggression have been shown by pharmacological and transgenic methods, which further identified a specific contribution of the dopaminergic system (Couppis and Kennedy, 2008; Aleyasin et al., 2018a).

Although the assumption of an implication of the NAc in reward-related aggression appears to contradict present findings, it is possible that such a relation is primarily associated with high levels of aggression, and due to the absence of a H-Agg phenotype in DA rats, an aggression-specific effect on neuronal activation might have remained undetected in this strain. Nevertheless, it remains unclear whether the reward-associated effects of aggression may share similar neurobiological mechanisms with the reward component of choice impulsivity.

In the DRN, the results for c-Fos expression differ between DD-task performance and RI tests and thus suggest a differential involvement of this brain region in impulsivity and aggression as well. While performance in the DD task increased neuronal activation in the DRN, this region was not affected by inter-male confrontations. However, the experiment in chapter 3 additionally investigated the number of neurons expressing the serotonin receptor 5-HT_{1A}, which were increased in the DRN in all behavioral groups. Combined results for c-Fos and 5-HT_{1A} thus demonstrate a role of the raphe nucleus in delayed reinforcement and in inter-male confrontations via the serotonergic system.

However, given that these changes in 5-HT_{1A} expression were not specific for impulsivity or aggression level, the observed effects might be subjected to other, more generalized aspects of behavior, motivational factors, or might be associated with separate aspects of impulsive and aggressive behavior such as reward-mediated processes.

In general, the 5-HT_{1A} receptor is the primary receptor subtype within the DRN (Mengod et al., 2015). It is mainly localized on neurons that synthesize the neurotransmitter serotonin and thus it functions as an autoreceptor on these serotonergic cells (Celada et al., 2001; de Boer et al., 2009). The activation of the 5-HT_{1A} receptor by serotonin results in negative feedback control and a reduction in serotonin synthesis or release because of the inhibitory influence of this receptor subtype (Mengod et al., 2015).

With regard to delayed reinforcement, activation of 5-HT_{1A} by selective agonists have previously shown to either increase impulsive choice behavior (Evenden and Ryan, 1999; Winstanley et al., 2005; van den Bergh et al., 2006a; Stanis et al., 2008; Blasio et al., 2012) or decrease it in some cases (Bizot et al., 1999; Zaichenko et al., 2013). The enhanced receptor level for H-Imp individuals of the present work is thereby in accordance with former reports, while the increase for L-Imp rats is consistent with the latter studies. However, the pharmacological compounds used in these earlier studies were administered systemically and not locally into the DRN which presumably activated 5-HT_{1A} receptors in other brain regions as well. Nevertheless, certain subpopulations of DRN neurons have been found to respond for large rewards, whereas others are rather activated by small rewards (Nakamura, 2013). This might explain a comparable increase in receptor expression in individuals with different impulsive phenotypes, and it demonstrates a potential involvement of the DRN and the serotonergic system in the modulation of both impulse control and delay aversion. Other studies with focus on serotonergic DRN neurons were additionally able to associate neuronal activation with the acquisition of expected and unexpected rewards (Luo et al., 2015; Li et al., 2016) and increased waiting for rewards (Miyazaki et al., 2014; Fonseca et al., 2015; Li et al., 2016), suggesting a role of the DRN in both reward- and delay-based decision making processes.

The DRN and specifically its serotonergic influence has also been previously linked to aggressive behavior. Confrontations in an RI test, for example, increased serotonergic neurotransmission (Mark et al., 2019) and enhanced c-Fos levels in H-Agg individuals (van der Vegt et al., 2003; Mark et al., 2019). Moreover, 5-HT_{1A} agonism within the DRN inhibited aggressive behavior in rats and thus the 5-HT_{1A} receptor was attributed an anti-aggressive influence (de Boer et al., 2000; van der Vegt et al., 2003). The enhanced receptor expression in L-Agg DA rats might reflect such an influence for keeping aggression levels at a minimum, but the simultaneous increase for M-Agg individuals does not support this view. Here, too, possible explanations are (1) the absence of a H-Agg group which might mask differences between phenotypic extremes observed in other rodent strains (van der Vegt et al., 2003; Mark et al., 2019) or (2) a differential role of neuronal subpopulations within the DRN as described for impulsive choice behavior which might likewise reflect the complexity of the serotonergic system and its receptors in behavioral regulation.

Still, the observed similarities for impulsivity and aggression groups of the present work indicate shared neurochemical processes mediated by the 5-HT_{1A} receptor, but the explicit implications of the DRN and the 5-HT_{1A} receptor in the regulation of impulsive-aggressive behavior remains to be resolved.

5.3. Neuronal networks in choice impulsivity

To further investigate potential differences within the behavioral spectrum of impulsive choice behavior at the neurochemical level in male DA rats, the expression levels of the serotonin receptor 5-HT_{1A} and the dopamine receptor D2 were analyzed and compared for L- and H-Imp individuals in chapter 4.

Altogether, results from chapter 3 and 4 demonstrate that DA rats with different levels of impulsivity have a very similar neuronal network which includes frontal cortex regions, the nucleus accumbens (NAc), the ventral tegmental area (VTA) and the DRN (Fig. 5.1). Findings from chapter 4 thereby expand the network involved in DD task performance by several brain regions which did not show changes in neuronal activation in chapter 3. Moreover, the results of chapter 4 demonstrate that choice impulsivity is not mediated by the D2 or 5-HT_{1A} receptors in BLA or SNc, which supports the current finding that BLA neurons were not activated by the DD task, nor were there any impulsivity-specific effects in this brain region.

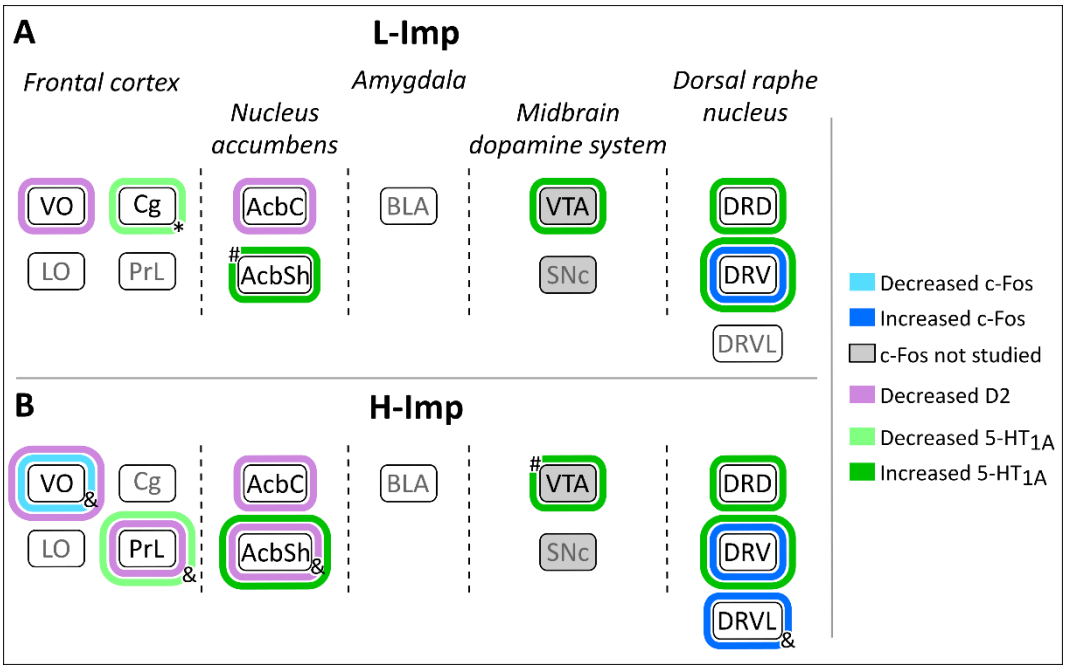

Figure 5.1 | Summary of the main effects of delay-discounting performance on c-Fos, 5-HT$_{1A}$ and D2 receptor expression in the brains of low- (L-Imp) and highly-impulsive (H-Imp) DA rats. Examined brain regions were the ventral (VO) and lateral orbitofrontal (LO) cortices, anterior cingulate cortex (Cg) and prelimbic cortex (PrL), nucleus accumbens core (AcbC) and shell (AcbSh), basolateral amygdala (BLA), ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and dorsal (DRD), ventral (DRV) and ventrolateral (DRVL) subregions of the dorsal raphe nucleus. Brain regions in which no changes were detected are denoted in gray.
*Significant statistical difference between impulsivity groups.
[&]Significant statistical difference compared to control, but no difference between impulsivity groups.
[#]Non-significant trend compared to control.

The only significant difference between both impulsivity levels was observed within the anterior cingulate cortex (Cg), a medial prefrontal subregion. In the Cg, the expression level of 5-HT$_{1A}$ was reduced in individuals with low impulsive choice compared to H-Imp individuals.
In the frontal cortex, the 5-HT$_{1A}$ receptor mainly functions as postsynaptic heteroreceptor on non-serotonergic neurons and exerts an inhibitory effect on these cells (Barnes and Sharp, 1999; Aznar et al., 2003). A reduction in receptor availability and thus a reduction of the inhibitory influence of this receptor subtype potentially increases neurotransmission within the Cg in male DA rats. Hence, present results indicate an involvement of the Cg subregion in the maintenance of low levels of choice impulsivity and thus in promoting self-control mediated via the serotonergic system.
To my current knowledge, neuronal activation in the Cg has not been investigated earlier in the context of delay discounting and pharmacological manipulations of the 5-HT$_{1A}$ receptor in the mPFC were shown to have no influence on impulsive choices (Yates et al., 2014). Previous studies using selective lesions of the Cg also reported no effect on choice behavior (Cardinal et al., 2001; Rudebeck et al., 2006). Hence, the present work is the first to associate delayed reinforcement with the Cg subregion. However, this area was more often linked to other forms of decision-making including effort-based choice (Walton et al., 2003; Schweimer and Hauber, 2005; Rudebeck et al., 2006) or performance in a 5-CSRTT (Muir et al., 1996; Passetti et al., 2002; Chudasama et al., 2003). More specifically, in effort discounting procedures, the individual is required to exert physical effort for a large reward, such as climbing a barrier or pressing a lever several times. Lesions of the Cg were reported to diminish the willingness to expend such effort, indicating an involvement of this area in cost-benefit evaluations

(Walton et al., 2003; Schweimer and Hauber, 2005; Rudebeck et al., 2006). The large reward is thereby linked to the response cost of energy consumption being dissociable from the processing of temporal costs specifically relevant for delayed reinforcement (e.g., delay tolerance) (Rudebeck et al., 2006; Floresco et al., 2008).

The 5-CSRTT, on the other hand, is a behavioral task which is used to assess measures of attention, motivation and motor impulsivity (inhibitory control). In order to obtain a reward, a subject is required to accurately select one of five spatial locations signaled by a visual stimulus. In earlier studies, lesions of the Cg were found to impair choice accuracy in the 5-CSRTT, i.e., reduce the relative number of correct responses, which reflects a measure of the subjects' attentional performance (Passetti et al., 2002; Chudasama et al., 2003). However, this parameter was additionally altered under varied task conditions which made the light stimulus temporally unpredictable (Passetti et al., 2002; Chudasama et al., 2003). Beyond a role in attention, the authors interpreted the observed behavioral effects as a disruption in temporal organization of behavior, or in other words, they suggested an involvement of the Cg in the processing and execution of events that need to be performed in sequence. Given that the temporal ordering of events is also relevant for the performance in a DD procedure, the involvement of the Cg in temporal processing reported by Chudasama et al. (2003) and Passetti et al. (2002) might have been a crucial factor for the observed differences between L- and H-Imp DA rats of the present work. Although highly speculative, the discrepancies between present findings and earlier research on the implication of the Cg in choice impulsivity (Cardinal et al., 2001; Rudebeck et al., 2006; Yates et al., 2014) might be explained by a supporting role of the Cg in above mentioned timing abilities. Hence, the Cg might not be the primary brain region mediating such processes during delayed reinforcement but it might provide a modulatory influence presumably via the serotonergic system, because 5-HT_{1A} receptor expression was affected in DA rats whereas neuronal activation patterns remained unchanged.

Another interesting aspect of the present findings is the reduction of 5-HT_{1A} levels in the frontal cortex in contrast to its increase in the other brain regions which showed a change in 5-HT_{1A} expression. It may be possible that changed expression levels within cortical areas are thereby linked to changes in serotonergic neurotransmission in the DRN, given the connections of these areas (Celada et al., 2001; Soiza-Reilly and Commons, 2011; Ogawa et al., 2014; Muzerelle et al., 2016). In particular, the increased inhibitory effect of 5-HT_{1A} autoreceptors in the DRN and thus a concomitant decrease in serotonin release in its cortical projection areas might have affected receptor dynamics by reducing 5-HT_{1A} receptor levels within the frontal cortex due to a diminished serotonergic input. For the verification of such an assumption, it would be necessary to combine a DD task with connection measures and the identification of neuronal subtypes, as previously done for 5-HT_{1A} mRNA levels by Meda et al. (2019) in cortico-amygdala or cortico-striatal pathways.

In the PrL, the second medial prefrontal subregion analyzed in the present work, the D2 receptor showed a tendency towards an impulsivity-specific effect with reduced receptor levels for H-Imp rats. These findings potentially implicate the mPFC in mechanisms regulating delay aversion specifically mediated via the dopaminergic system. Considering the inhibitory nature of the D2 receptor on non-dopaminergic neurons when acting as a postsynaptic heteroreceptor (Beaulieu and Gainetdinov, 2011; Ford, 2014), the observed reduction in receptor expression levels presumably increased neuronal signaling within the PrL which in turn contributed to an enhanced intolerance towards delays. Present findings are consistent with an effect of D2 receptor blockade by selective antagonists in the mPFC which also resulted in increased choice impulsivity in DD procedures (Pardey et al., 2013; Yates et al., 2014). However, Yates et al. (2014) observed a similar increase in choice impulsivity after infusion of a D2 agonist. These conflicting results in the pharmacological studies might be based on

methodological factors such as the design of the inter-temporal choice task (DD task vs. adjusting-delay procedure) or the size of the investigated brain area which, for example, included prelimbic and infralimbic cortices in the study by Yates et al. (2014), but only encompassed the PrL in the study by Pardey et al. (2013). Nevertheless, although more detailed research is needed, the present work appears to provide a differential role for the Cg in promoting self-controlled choices and the PrL in regulating delay aversion, both including monoaminergic transmitter systems.

With regard to the role of the OFC vs. the mPFC, current results on monoaminergic receptors lead to a different view than those for neuronal activation patterns discussed above in section 5.2.

While altered c-Fos expression levels within the VO subregion suggest a primary involvement of the OFC in choice behavior, the impulsivity-specific effects found for the Cg in chapter 4 and a corresponding trend for the PrL rather indicate a predominant role for mPFC subregions, at least with involvement of 5-HT_{1A} and D2 receptors. This inconsistency is also evident in the literature (see, for example, Cardinal, 2006; Cardinal et al., 2001; Winstanley et al., 2006b).

Nevertheless, in male DA rats, neither the OFC nor the mPFC alone appear to mainly affect impulsive responding, but present results illustrate a complex contribution of various cortical subregions to DD task performance, which most likely include reward- and delay-related aspects of behavior, different monoaminergic transmitter systems as well as a temporal component such that different brain regions are potentially recruited at varying time points of the DD task.

5.4. Neuronal networks in territorial aggression

Findings from chapter 3 demonstrate that DA rats with different levels of aggression show overlaps in their neuroanatomical network in the LO, PrL, NAc and DRN. Significant differences within the networks of L- and M-Agg rats occurred in VO and Cg subregions and the BLA. Thus, the current work provides evidence for distinct circuits that underlie low and medium aggression profiles.

Especially the frontal cortex appears to be involved in regulatory mechanisms for the display of aggressive behavior as well as its suppression, as shown by regionally-specific changes in neuronal activation patterns in chapter 3 (Fig. 5.2).

Involvement of the frontal cortex in territorial aggression has already been described by earlier studies, although reported findings are mixed. Several studies found an association between heightened aggression and increased c-Fos expression in OFC and mPFC (Halász et al., 2006; Haller et al., 2006; Toth et al., 2012). However, Nehrenberg et al. (2013) reported decreased c-Fos levels in mice selected for high aggression. Other examples of negative relations or no effect were shown by studies using lesions or optogenetic manipulations. Lesions of the OFC or the Cg subregion were reported to enhance aggressive behavior or have no effect on aggression, respectively (Rudebeck et al., 2007). Optogenetic activation of the OFC had no effect on aggression while activation of the mPFC decreased offensive behavior (Takahashi et al., 2014).

Interestingly, previous studies mainly reported effects for aggressive individuals, while neuronal activation patterns in L-Agg individuals were unchanged and thus resembled baseline level (Haller et al., 2006).

In this regard, present findings substantially differ from previous research due to the observed decrease in c-Fos levels in L-Agg DA rats.

One reason for these discrepancies might be the absence of a H-Agg phenotype in male DA rats, as previously mentioned in section 5.2., because potential changes in neuronal activation could remain undetected when these are primarily associated with enhanced aggression. However, this assumption does not explain significant changes for L-Agg individuals compared to control rats in the present work.

With regard to the relationship, low levels of aggression along with neuronal inhibition reflects a similar positive link as found between high aggression and neuronal activation. The direction of effects, however, differs, and this may result in different and conflicting interpretations for the involvement of the frontal cortex in aggression control. More explicitly, an inhibition of frontal subregions in L-Agg rats suggests an involvement in the suppression of aggressive behavior, whereas an activation in H-Agg individuals indicates that these areas promote the display of aggressive acts. Given that neuronal inhibition also occurred for M-Agg DA rats in the OFC, it is possible that certain subregions of the frontal cortex initiate aggressive behavior through inhibitory control. In total, present and previous evidence demonstrates a complex role of the frontal cortex in the regulation of species-specific adaptive aggression (also see: Miczek et al., 2015; Takahashi et al., 2014).

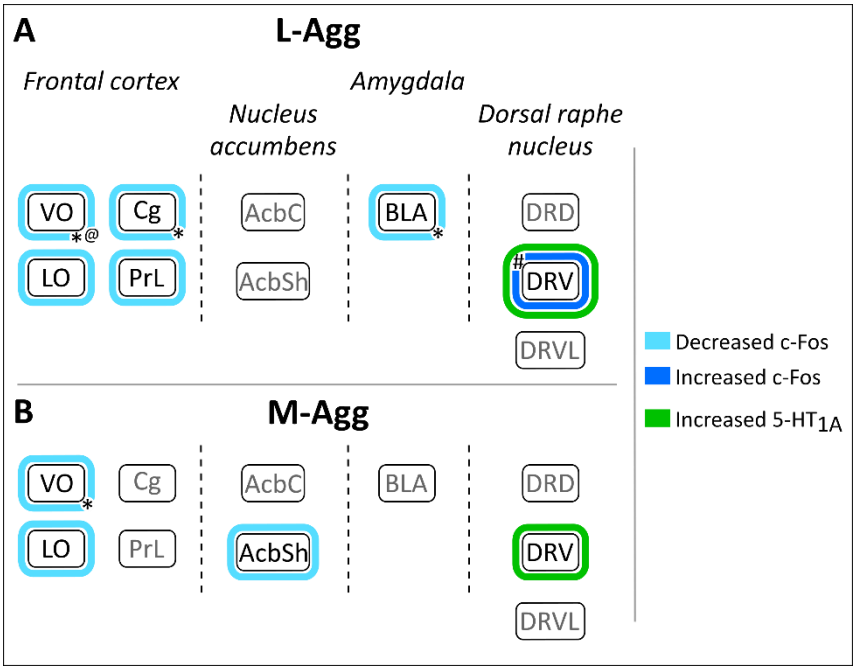

Figure 5.2 | Summary of the main effects of resident-intruder confrontations on c-Fos and 5-HT$_{1A}$ receptor expression in the brains of low- (L-Agg) and medium-aggressive (M-Agg) DA rats. Examined brain regions were the ventral (VO) and lateral orbitofrontal (LO) cortices, anterior cingulate cortex (Cg) and prelimbic cortex (PrL), nucleus accumbens core (AcbC) and shell (AcbSh), basolateral amygdala (BLA), and dorsal (DRD), ventral (DRV) and ventrolateral (DRVL) subregions of the dorsal raphe nucleus. Brain regions in which no changes were detected are denoted in gray.

*Significant statistical difference between aggression groups.

@Significant statistical difference compared to M-Agg group, but not to control group.

#Non-significant trend compared to control.

Findings from chapter 3 additionally demonstrate that aggression-specific effects in DA rats, as shown by significant differences between L- and M-Agg rats, are attributed to VO and Cg subregions. The inhibition of LO and PrL, on the other hand, was not specific for aggression level which potentially underlies other factors associated with inter-male confrontations, such as social arousal. A study by Halász et al. (2006), for example, examined the influence of two forms of inter-male confrontations, namely a RI paradigm and a psychosocial test, on c-Fos expression levels in the frontal cortex. For the psychosocial test, the opponents were spatially separated to allow olfactory and visual inspections, but no physical contact. The authors found increased c-Fos levels after both tests in several frontal

subregions including those investigated in the present work (VO, LO, PrL, Cg). In an earlier study by the same research group, similar results were obtained for the DRN which were suggested to be due to social arousal rather than aggressive behavior (Haller et al., 2005). Thus, the observed inhibition of LO and PrL in the present work might be induced by social arousal as well.

This interpretation might also be valid for the DRN of DA rats for which no group differences were present with regard to the number of neurons expressing the 5-HT_{1A} receptor. This receptor is not only expressed as inhibitory autoreceptor in the DRN, but it is widely distributed in raphe projection areas such as frontal cortex, septum, striatum, hippocampus, hypothalamus and amygdala (Barnes and Sharp, 1999; Aznar et al., 2003; Santana et al., 2004; Millan et al., 2008). An increase in 5-HT_{1A} expression, as found for the DRV subregion in chapter 3, might inhibit serotonin synthesis when acting on 5-HT neurons in the raphe nucleus (also see: Celada et al., 2001; de Boer et al., 2009). In contrast to present findings, Haller et al. (2005) reported that increased activation of DRN 5-HT neurons in particular reduced aggressive behavior, although, as mentioned above, the authors reported no aggression-specific effect when the entire DRN was considered. Accordingly, certain subpopulations of DRN neurons might be involved in regulatory control of aggression also including 5-HT_{1A} activity. In accordance with this possibility, pharmacological studies have shown that direct injection of 5-HT_{1A} agonists into the DRN decreased the level of aggression (Mos et al., 1993; van der Vegt et al., 2003).

In view of this, the current approach might have failed to detect important changes in 5-HT_{1A} expression due to the sole quantification of neuron number. A follow-up study with an additional assessment of the receptor expression level by use of receptor density as a parameter might reveal a more detailed pattern of alterations of the 5-HT_{1A} receptor in the DRN and its projection sites, similar to chapter 4 of the present work which addressed this issue in the context of choice impulsivity.

With regard to aggression-specific effects, the BLA was the third brain area to show changes between L- and M-Agg rats. The effect was similar to the frontal cortex, in that an inhibition of the BLA was associated with low aggression. Its connection with cortical areas might, in fact, be important for the regulatory processes mediated by the BLA. Through intense reciprocal projections to OFC and mPFC, the amygdala is thought to process relevant information about emotional stimuli and social behavior to keep aggressive behavior in an adaptive range (Siever, 2008; Felix-Ortiz and Tye, 2014; Takahashi et al., 2014; Biro et al., 2017).

While in some cases anti-aggressive effects are suggested to underlie inhibitory inputs from the frontal cortex (for review see: de Almeida et al., 2015; Nelson and Trainor, 2007), a study by Nehrenberg et al. (2013) has shown that c-Fos expression levels were increased in both the frontal cortex and the amygdala of mice selected for low levels of aggression compared to their H-Agg counterparts. Thus, instead of being inhibited, the authors assumed that different amygdalar nuclei are activated through excitatory glutamatergic projections in low aggressive individuals. Although, in contrast to the latter study, present findings show decreased neuronal activity of the BLA in L-Agg individuals, they support the notion of an inhibitory influence exerted by the frontal cortex.

Hence, irrespective of the direction of effects, the existing evidence highlights the importance of the BLA in the modulation of aggressive behavior.

5.5. Conclusion and future perspectives

All in all, the present work has shown that cortical structures are highly relevant for the regulation of choice impulsivity as well as territorial aggression in male DA rats. Although behavioral results point towards a dissociation between these two traits, the OFC appears to be implicated in both increased

intolerance towards delays as well as diminished aggressive behavior, demonstrating an overlap in the neuroanatomical basis of these behaviors.

The suppression of offensive behavior can also be attributed to mPFC and BLA function, incorporating a subcortical structure in the control of species-specific, adaptive aggression. Furthermore, current findings demonstrate a differential contribution of the medial prefrontal subregions Cg to the promotion of self-controlled choices including the serotonin 5-HT_{1A} receptor and the PrL to the regulation of delay aversion mediated via dopamine D2 receptor activity.

In addition, present data suggest that the DRN and its serotonergic neurons are an important neural substrate for the performance of inter-temporal choice procedures as well as inter-male confrontations. Nevertheless, it remains to be resolved whether the observed effects specifically underlie varying behavioral levels or are linked to single behavioral components such as reward-related processes, motivational factors or social aspects of behavior. Moreover, it needs to be clarified whether these DRN-mediated mechanisms are shared between impulsivity and aggression.

Overall, current findings give a general overview of relevant brain areas implicated in impulsivity and aggression in male DA rats which could be used as a basis for subsequent investigations on the precise involvement of specific neuronal populations located within frontal cortical subregions and the dorsal raphe nucleus.

At the behavioral level, future studies that focus on different combinations of behavioral tasks, such as the RI test scheme together with paradigms that separately evaluate delay or reward magnitude sensitivities, could identify potential relationships between single behavioral components underlying choice impulsivity and territorial behaviors, such as reward-related processes. Second, given that the present work used a more classical immunohistochemical staining method following the completion of behavioral testing, future research could aim at identifying the involvement of relevant brain regions at different stages of the behavioral paradigms by use of *in vivo* techniques. Such approaches could involve optical methods, including optogenetics or fiber photometry, to directly manipulate or monitor changes in neuronal activity during task performance in behaving animals. These methods offer advantages with regard to temporal precision, the targeting of specific types of neurons and they are applicable to several tasks because induced optogenetic effects are reversible (for review see: Carr et al., 2018). Chemogenetic approaches represent another promising research tool for precise manipulations of neurons or neuronal circuitries, similar to optogenetic techniques but without the necessity of optical fiber implantations (for review see: Atasoy and Sternson, 2018). This would avoid potential restrictions for the animals' movements during extensive fighting periods or social contacts in inter-male confrontations.

Incorporating these methods with receptor detection by fluorescence staining or the use of connectivity measures, such as tracing techniques, in future behavioral studies would help to further dissect the shared neuroanatomical and -chemical components that underlie choice impulsivity and territorial aggression.

6. References

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List of manuscripts and explanation of contributions

The present thesis is based upon the manuscripts enlisted below. Contributions of the candidate to a multi-author manuscript are presented in percent of the total work load.

Chapter 2 - Relationship between impulsive choice and territorial aggression in male Dark Agouti inbred rats
Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

Experimental concept and design:	ca. 60 %
Experimental work and/or acquisition of (experimental) data:	ca. 100 %
Data analysis and interpretation:	ca. 80 %
Preparation of Figures and Tables:	ca. 100 %
Drafting of the manuscript:	ca. 50 %

Chapter 3 - Comparative analysis of c-Fos expression levels in impulsive choice behavior and territorial aggression in male Dark Agouti rats
Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

Experimental concept and design:	ca. 70 %
Experimental work and/or acquisition of (experimental) data:	ca. 100 %
Data analysis and interpretation:	ca. 90 %
Preparation of Figures and Tables:	ca. 100 %
Drafting of the manuscript:	ca. 80 %

Chapter 4 - Differential changes in dopamine D2 receptor and serotonin 5-HT_{1A} receptor expression in the brains of Dark Agouti rats trained in a delay-discounting task
Johanna Brigitte Artelt-Radziejewski and Ursula Dicke

Experimental concept and design:	ca. 80 %
Experimental work and/or acquisition of (experimental) data:	ca. 100 %
Data analysis and interpretation:	ca. 100 %
Preparation of Figures and Tables:	ca. 100 %
Drafting of the manuscript:	ca. 100 %

Erklärung an Eides Statt

gemäß § 7 Abs. 7 der Promotionsordnung der Universität Bremen vom 08.07.2015 für den Fachbereich Biologie/Chemie

Hiermit erkläre ich, Johanna Brigitte Artelt-Radziejewski, dass die eingereichte Dissertation mit dem Titel „Impulsive-aggressive phenotypes in male Dark Agouti rats: Neuronal correlates and effects on monoaminergic receptors“ von mir selbstständig angefertigt und verfasst wurde. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel verwendet und wörtlich oder inhaltlich entnommene Stellen aus den angegebenen Quellen habe ich als solche kenntlich gemacht. Des Weiteren erkläre ich, dass die zu Prüfungszwecken beigelegte elektronische Version der Dissertation mit der abgegebenen gedruckten Version identisch ist.

(*Johanna Brigitte Artelt-Radziejewski*)

Bremen, Februar 2022