“Differential metabolic recruitment of cognitive, emotional and modulatory brain regions in infant and adolescent rats undergoing two-way active avoidance training.”

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1. Introduction

1.1. Basics of learning and memory: Behaviorism vs. Cognitivism

"Learning" enables individuals to adapt to changes in their environment. A general definition of "learning" describes a process during which long-lasting modifications in the behavioral potential occur as a result of experience. In the brain, this process requires that information is appropriately encoded and, in this way, prepared for storage. In contrast, "memory" is defined as the record of the experience that underlies learning. "Memory" comprises storage, consolidation, retention and recall, i.e. retrieval, of information (Anderson, 2000).

During the beginning of psychology research, the investigation of learning was accomplished by behaviorist approaches, whereas the search for memory was tightly linked to cognitive approaches. Taking advantage of animal research, behaviorists hypothesized that understanding any aspects of human behavior depended on understanding how that behavior was acquired. They did not pay attention to what might be happening in the brain or mind of an organism. In contrast, early cognitivists emphasized understanding the functioning of the mature cognitive system rather than the underlying learning processes that shaped the system. Focusing on the human brain, they argued that complex mental processes play an important role in the shaping of behavior. Thus, traditional cognitivism did not consider the objectively observable stimuli but rather the cognitive representation of the stimulus pattern, i.e. stimuli as coded or "proximal stimuli" as triggers for a response (Baltes and Reisenzein, 1985). Nowadays, these two different lines of research have converged in the field of the Neurosciences that investigate the neurobiological substrates of behaviors – among them learning and memory.

In laboratory research, animal learning is achieved by classical and instrumental conditioning, which enable organisms to anticipate biologically significant stimuli. The resulting adaptive reactions are called conditioned responses.

Thus, during ***classical conditioning***, a neutral stimulus (NS), e.g. a light

or tone, is presented to an animal together with an unconditioned stimulus (UCS), e.g. food or foot-shock. Initially, only the UCS causes an unconditioned response (UR), e.g. salivation after food presentation or freezing after foot-shock presentation. If the NS and the UCS are presented contiguous[1] and contingent[2] enough over a certain number of trials, the NS – which now became the conditioned stimulus (CS) – itself will evoke the identical response, which is now called conditioned response (CR). Classical conditioning is the simplest way to achieve association learning. Anyhow, it is rather considered to provoke reflex-like behaviors (Anderson, 2000). One of the most frequently applied paradigms in experimental animal research is fear conditioning (Fig. 1).

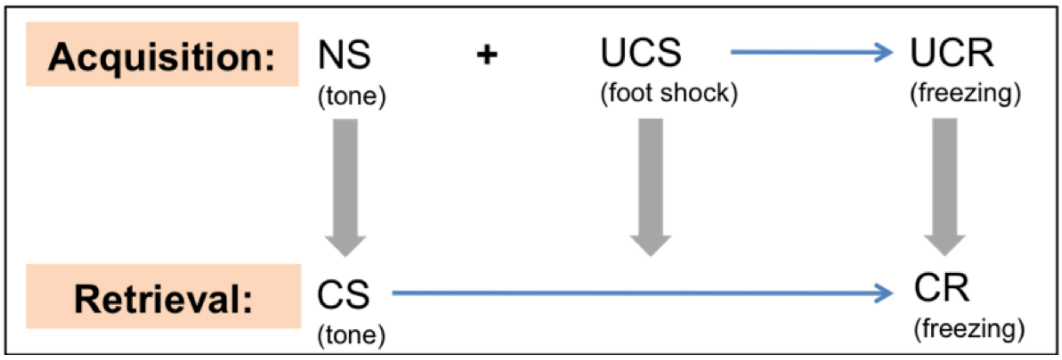


Figure 1: Process of CS-UCS association during classical (fear) conditioning. During the acquisition period, the NS is presented together with the UCS. The animal learns to anticipate the UCS when the NS is presented. The NS, thereby, becomes a CS. Anticipation of the UCS (although not presented any more) provokes the behavioral response, which is now called conditioned response (CR).

A more complex type of learning is achieved by ***instrumental conditioning***, which is synonymously used with the term "operant conditioning". This terminological distinction has emerged from different perspectives on the learning paradigms. The term "instrumental" relates to the experimental prerequisites meaning that an apparatus or environmental setup is needed to create a situation where a certain behavior can be performed. Edward L. Thorndike – the first to systematically study instrumental conditioning in cats – thought that the initially random behavior is abandoned over the trials as reinforcers

[1] contiguous, contiguity: concerns the temporal proximity of CS and UCS
[2] contingent, contingency: concerns the probability of the first stimulus to predict the occurrence of the second stimulus

strengthen the stimulus-response connection (Law of effect, Thorndike, 1898, 1998). The term “operant” simply focuses on the fact that a certain behavior (=operant) is needed to respond to a stimulus (Skinner, 1938).

Basically, stimuli can be pleasant or unpleasant; they can be supplied to or removed from the situation after the behavioral response. Thereby, stimuli can reinforce behaviors[3]. According to type and contingency of the stimulus on the response, instrumental-conditioning tasks can be basically assigned to four different categories (Tab. 1).

type of stimulus and contingency on response	**stimulus** ↙ **pleasant/ appetitive** (positive)	↘ **unpleasant/ aversive** (negative)
stimulus given if behavior is performed	**positive reinforcement** e.g. pellet supply after lever press	**punishment** e.g. electric shock after lever press
stimulus removed if behavior is performed	**omission** e.g. removal of pellet during grooming	**negative reinforcement** e.g. avoidance of an electric foot shock by lever press or changing to the other shuttle box compartment

Table 1: Types of stimulus and contingency on response. Adapted from Lefrancois (2003) and Anderson (2000).

1.2. The two-way active avoidance task

The two-way active avoidance (TWA) task is an instrumental conditioning paradigm. It utilizes negative reinforcement to provoke a CR. Basically, the animals learn to avoid an unpleasant foot-shock (the UCS) as a consequence of CS presentation (Tab. 1).

For TWA training, shuttle boxes consisting of two incompletely separated compartments and a floor grid are used (Fig. 2). The electrical foot-shock is always delivered in the compartment where the animal is staying after a defined resting period (the ‘inter-trial interval’). During the initial trials, the animal displays unconditioned reactions meaning that it simply escapes from the unsafe compartment to the opposite (safe) compartment in

[3] thus, during instrumental conditioning, learning depends on the contiguity (temporal proximity) and contingency (predictive power of the response) of response and reinforcer

response to the foot-shock (=escape reaction). Later on, the animal escapes the unsafe compartment already in response to the CS presentation. This pre-term-escape is called “avoidance reaction” or conditioned avoidance response. Frequent and regular performance of the avoidance reaction indicates that the animal has learned an association between the antecedent configuration of stimuli (CS-UCS) and the response.

The description given above implies that beside the avoidance problem itself, the assembly of a shuttle box creates additional difficulties for the animal that have to be overcome. The escape/avoidance route is bi-directional rendering the task spatially more complex than one-way avoidance tasks and causing the conflict to enter the opposite compartment where the animal experienced the aversive foot-shock before (Olton, 1973; Savonenko et al. 1999a). Thus, the TWA task requires a number of cognitive as well as emotional processes and is considered more comparable to complex learning occurring in higher developed mammalian species than classical or other instrumental conditioning paradigms are.
With respect to comparative studies, it is an advantage of the TWA paradigm that the evoked responses are not antagonistic to biologically inherent reactions. Flight reactions are naturally occurring in rats, which experience aversive sensory stimuli. This is, e.g., not the case in other negative reinforcement paradigms like ‘lever-press avoidance’[4].

The classical theory of avoidance learning is the ***two-process theory*** suggested by Mowrer (1939, 1947) and elaborated by Miller (1948). It states that the first step of active avoidance learning is the classical conditioning phase, which is provoked by the pairing of CS and UCS and, consequently, results in fear as a CR. The second phase utilizes the potential of this fear to provoke an instrumental response.

[4] During lever-press avoidance, the animal has to press a lever to interrupt or avoid a foot-shock. Thus, it cannot utilize its natural UCR and simply take flight.

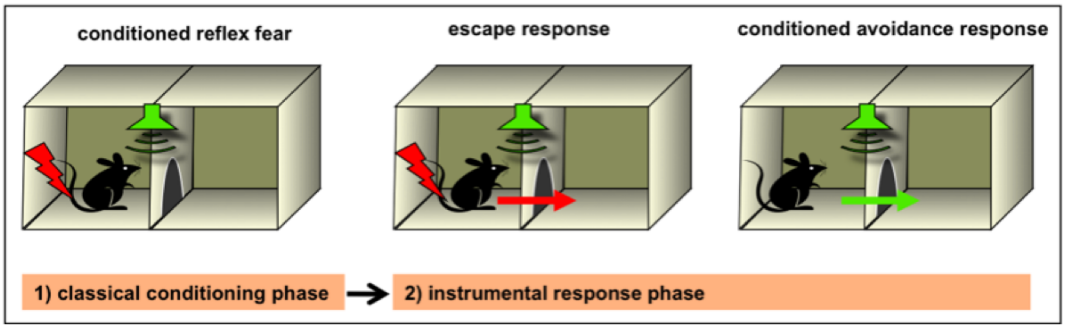


Figure 2: Principle of the shuttle-box (adapted from Abraham et al., 2005). During the classical conditioning phase, the animal develops fear of the UCS, which is a foot-shock. During the instrumental response phase, it realizes that the UCS can not only be ceased (escape response) but also be avoided (avoidance response). Stepwise, avoidance becomes the preferred behavior (Miller and Konorski, 1969; Mowrer, 1939; Miller, 1948).

Although Mowrer and Miller are traditionally considered the first to introduce a consistent theory about the formation of avoidance behavior, there are two other scientists who earlier submitted a similar idea. Thus, already in 1928 – only 24 years after Ivan P. Pavlov was awarded the Nobel Prize for his research about classical conditioning of reflex responses – two Polish physiologists, Jerzy Konorski and Stefan Miller (another Miller than the one cited above) described a "conditional reflex type II", which was provoked by instrumental conditioning using negative reinforcement (Miller and Konorski, 1928, 1969; Windholz and Wyrwicka, 1996). Regarding the underlying mechanism of formation, they hypothesized that inborn reactions of dislike and pain, which primarily consist of peripheral autonomous processes are evoked by aversive stimuli. They called this endogenous component "fear or anxiety"[5] (first component). Consequently, the peripheral autonomous processes trigger proprioceptive feedback, which is the drive to motivate the organism for avoidance behavior (second component).

However, it was Neil E. Miller who provided consistent experimental evidence for the two-process theory. In his seminal study, he demonstrated that the fear, which animals acquired by application of foot-

[5] Although the terms "fear" (a distressing emotion caused by a threat in form of a specific simple or compact sensory stimulus) and "anxiety" (a normal reaction to stress that, however, can become pathological) are clearly defined, they are still frequently used synonymously in the literature. This is understandable because in practice, the stimuli and symptoms overlap as soon as a situation becomes complex.

shocks was sufficient to develop a new behavioral (avoidance) strategy during the following instrumental procedure without the delivery of new shocks[6] (Miller, 1948). The study generated some very important conceptual ideas for the development of avoidance strategies: (I) In contrast to innate, i.e. primary or inborn drives like hunger, thirst or sex, ***fear is a readily acquirable drive***, which can be become very strong. (II) The arising ***motivation is to reduce the fear*** by the development of new behavioral strategies (=learning). (III) The ***fear-reduction serves as reinforcement***[7].

By this definition, avoidance learning is categorized as aversively motivated because initially the individual is punished. However, once an aversive outcome is successfully avoided, the individual may no longer experience punishment and avoiding the aversive outcome results in relief, which could be a reward in itself (Tab. 2; Kim et al. 2006).

emotions evoked by type and time of stimulus application	**stimulus**	
	positive (pleasant)	**negative** (unpleasant)
stimulus given if behavior is performed	**pleasure desire** (appetence excited)	**fear, anxiety, pain** (aversion excited)
stimulus removed if behavior is performed	**anger, rage sorrow, depression** (appetence inhibited)	**relief** (aversion inhibited)

Table 2: Emotions and behavioral drive states (gray) evoked by type and time of stimulus application. The scheme illustrates that the stimulus-response table (Tab. 1) can be similarly arranged applying higher-order psychological constructs. Compiled after Baltes and Reisenzein (1985), Miller and Konorski (1928, 1969) and Seymour et al. (2007).

[6] Cave: shock application and instrumental response training were applied in different apparatuses, not in the shuttle-box

[7] The drive-reduction theory has a long history in learning and memory research. A drive is conceptualized as an aversive stimulus that can become more extreme as the organism continues to be deprived. Hence, individuals survive because internal or acquired drives produce a strong desire to approach positive and avoid negative stimuli. Consequently, psychologists have proposed that behavior is organized around two mutually antagonistic motivational systems – an appetitive and an aversive system. Behaviors generated by the appetitive system bring the organism in contact with biological stimuli necessary for survival and reproduction, whereas behaviors mediated by the aversive system serve to remove the organism from harmful stimuli (Anderson, 2000).

1.3. Background of the present study

It is known that young rats show a poor TWA performance compared to adolescent and adult rats (Bauer, 1978; Izquierdo et al., 1975; Kudryashova, 2006). Recently, our group specifically elaborated that infant rats (P17-P21) are not able to establish adequate TWA behavior during shuttle-box training covering five subsequent training days with 50 trials every day (Schäble et al., 2007). This inability subsides with age. Thus, adolescent rats (P38-42) do already perform significantly better than the infant rats, and adult rats (P80-84), again, show a better performance than the adolescents (Fig. 3; Gruss et al., 2010).

Interestingly, when the infant rats were re-exposed to the shuttle box during adulthood and experienced the same TWA training like during infancy, they showed accelerated avoidance learning compared to their non-pre-experienced littermates (Fig. 3). Thus, although the infant rats failed to generate an adequate avoidance behavior, early experience seemed to engrave an enduring memory, which is of potential benefit for them (Schäble et al., 2007). More recently, Gruss et al. (2010) substantiated what the “early experience” necessarily has to cover to result in a behaviorally measurable benefit during adulthood. Accordingly, the contingent and contiguous presentation of the CS and UCS over five consecutive training days (50 trials/day) was necessary to produce the pre-experience effect, whereas application of CS, UCS or context solely as well as pseudo-training and simple handling did not lead to a significant acceleration of learning during adulthood (Fig. 3).

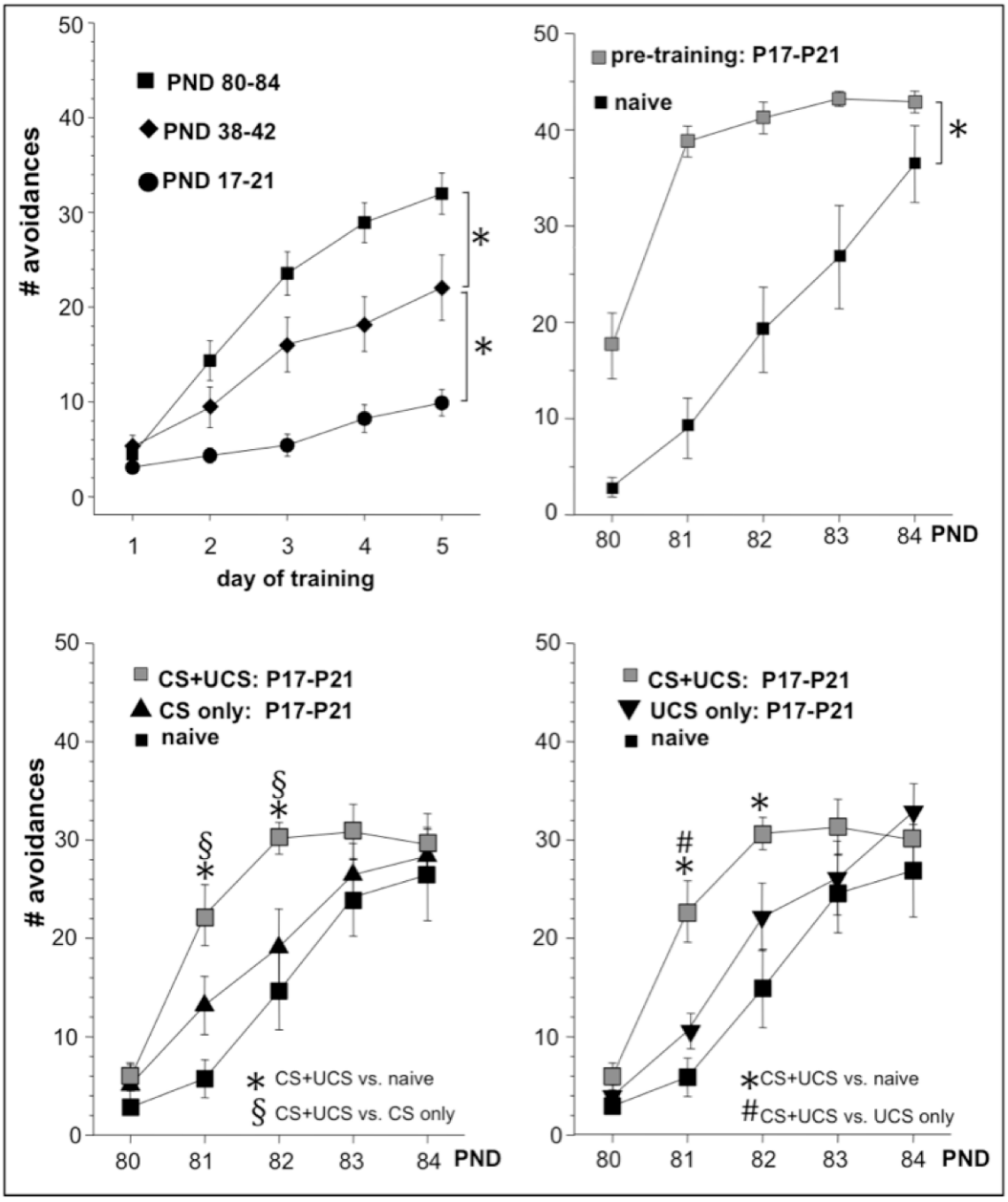


Figure 3: Effect of age and pre-experience on TWA performance. 1) TWA performance is age dependent (upper left). 2) Early TWA training leads to an accelerated response during adult re-training (upper right). 3) Presentation of the CS alone during infancy does not result in a significant learning improvement during adulthood (lower left). 4) Presentation of the UCS alone during infancy does not result in a significant learning improvement during adulthood (lower right). Adapted from Schäble et al., 2007 & Gruss et al., 2010.

In search of the neurobiological substrates underlying the ontogeny of TWA learning, Schäble et al. (2007) performed a number of pharmacological experiments.

Namely, prior to each training session, they systematically administered Haloperidol[8] to the infant rats. These animals took advantage of their pre-experience despite D2-receptor blockade indicating that interference with the dopaminergic system is not affecting the engraving of the "memory trace" at that early age.

However, when Haloperidol was administered to adolescent rats, they performed significantly worse when re-trained during adulthood compared to pre-trained animals without D2-receptor blockade. This effect is even more pronounced when Haloperidol was administered during adulthood (Gruss et al., unpublished; see also Carvalho et al., 2009; Reis et al., 2004).

Thus, memory storage obviously becomes more and more sensitive to dopaminergic mechanisms.

Taken together, the novel findings introduced above suggest, that the dopaminergic system in infant rats is immature, which could be one reason for their poor TWA-performance.

Despite their poor TWA performance, the infants obviously store information about the task, which can be retrieved later to facilitate learning during re-training.

From the findings introduced above, two major questions arose:

1) What do the infants learn?

2) Is the poor learning performance in infant rats correlated with the maturation of brain function?

[8] preferentially resulting in the blockade of D2-receptors

1.4. Aim of the present study

1.4.1. General objective of the study

There are a number of cortical, subcortical and brain stem regions that have been shown to play a role in different aspects of TWA learning in rats. This was historically – and is currently anew – accomplished primarily by lesion studies as well as pharmacological and electrophysiological experiments (Tab. 3).

The principle of these experiments is that inactivation or stimulation of a single brain region results in changes of TWA behavior compared to non-manipulated animals. Although this has led to a huge body of information, there are a number of problems arising from these methods. First, lesions on the regional level usually bring about varying neuronal damage resulting in non-identical, sometimes even contradictory behavioral outcomes (see e.g. Gray and McNaughton, 1983). Second, as stereotaxic operations are much more difficult in infant rats, the majority of these studies have been performed in adult animals. Finally, and most important, learning and memory processes strongly depend on intact regulation by modulatory states like attention and motivation, which are also affected by destruction of brain integrity.

Correlational strategies – in contrast to interventional strategies – allow the analysis of the intact brain. They allow the non-invasive detection of brain signals during rest or activity. The signals from many different recording sites can be explored for statistical dependencies. Moreover, the brain signals can be correlated with the behavioral performance.

At present, the only imaging method that allows the non-invasive measurement and quantification of ***regional brain activity at macroscale level in freely behaving animals*** is the ^{14}C-2-deoxyglucose (2-DG) method (Konkle and Bielajew, 2004; Sokoloff et al., 1977).

Table 3: Selection of studies analyzing the contribution of segregated brain regions to TWA learning (lesion, pharmacological treatment, electrical stimulation).

Manipulated/ investigated region	Type of manipulation/effect on TWA performance	References
Septum	huge number of lesion studies reviewed/ increase in performance more often than decrease or no change	Gray and McNaughton, 1983; review
Medial septum	ibotenic acid lesion/ fewer trials to reach criterion	Hepler et al., 1985
Medial septum	electrolytical lesion/ Increase in TWA performance	Torras-Garcia et al., 2003
Basal nc. of Meynert	electrical stimulation/ Improved acquisition of TWA performance	Montero-Pastor et al., 2004
Basal nc. of Meynert	ibotenic acid lesion on postnatal day 2/impaired acquisition of TWA performance durig adulthood	Sengstock et al., 1992
Hippocampus	huge number of lesion studies reviewed/ increase in performance more often than decrease or no change	Gray and McNaughton, 1983; review
CA3 region of hippocampus	ibotenic acid lesion/ Impaired TWA retention	Datta et al., 2005
CA3 region of hippocampus	PKA inhibition/ impaired TWA retention	Datta et al., 2009
CA1 and dentate gyrus	ibotenic acid lesion/ normal TWA retention	Datta et al., 2005
Dorsomedial PFC	electrolytic lesion/ impaired acquisition	Brennan et al. 1977
Medial PFC (incl. PL, excl. IL)	excitotoxic lesion by NMDA injection/ no effect on TWA performance	Lacroix et al., 1998
Prelimbic area	electrolytic lesions/ no effect on TWA performance	Fritts et al., 1998
Infralimbic area	electrolytic lesions/ increased TWA performance	Lacroix et al., 1998
Retrosplenial cortex	excitotoxic lesion by NMDA injection/ decrease in the acquisition of TWA performance	Lukoyanov and Lukoyanova 2006
Nc. accumbens, core and shell region	electrolytic lesion/ improvement in TWA performance	Gal et al., 2005
Nc. accumbens, core region	NMDA injection/no effect	Jongen-Rêlo et al., 2002
Nc. accumbens, shell region	NMDA injection/no effect	Jongen-Rêlo et al., 2002
Nc. accumbens, shell region	electrolytic lesion/ impairment in TWA performance	Gal et al., 2005
Caudate nucleus	cholinergic stimulation/ improvement in TWA performance	Prado-Alcala et al., 1984
Nc. parafascicularis	NMDA-infusion/ impaired TWA performance	Quiroz-Padilla et al., 2007

Continued on the next page.

Tab. 3 continued

Manipulated/ investigated region	Type of manipulation/effect on TWA performance	References
Lateral habenula	electrical stimulation/ impaired TWA performance	Shumake et al., 2010
Hypothalamus	no manipulation/ induction of CREB phosphorylation after TWA performance	Saha and Datta, 2005
Lateral hypothalamus	unilateral electrtolytic lesions/ impairment of TWA performance	Asdourian et al., 1977
Ventromedial hypothalamus	Electrolytic lesions/ Facilitation of TWA acquisition	Grossman, 1972
Amygdala	electrolytic lesion in infant (P10) vs. young adult (P60) rats/significant more trials to TWA criterion in P60 rats compared to controls but no difference in P10 rats	Molino, 1975
Central nc. of amygdala	electrolytic +/- ibotenic lesion/impairment of TWA performance; electrical lesion/impaired acquisition but not retention of TWA performance; increase of performance in originally poor performers	Sanchez Riolobos, 1986 Roozendaal et al., 1993 Choi et al., 2010
Basolateral nc. of amygdala	APV-infusion (i.e. NMDA receptor blockade)/ impaired acquisition of TWA performance	Savonenko et al., 2003
Basolateral nc. amygdala	bilateral Lesion/ impairment of acquisition of TWA performance	Segura-Torres et al., 2010
PPTg	electrical stimulation/ improved acquisition of TWA performance	Andero et al., 2007
SNc	MTPT lesion/ impaired TWA acquisition and performance	Da Cunha et al., 2001
VTA	MTPT lesion/ impaired TWA acquisition and performance	Da Cunha et al., 2001
VTA	dopamine depletion/ TWA performance abolished	Oades et al., 1987
VTA	electrical stimulation/ improved TWA performance	Shumake et al., 2010
DR, MR	systemic depletion of serotonin/ impaired acquisition and retention of avoidances during TWA learning when mild to moderate foot-shock intensities are applied	Galindo et al., 2008

Table 3: Selection of studies analyzing the contribution of segregated brain regions to TWA learning (lesion, pharmacological treatment, electrical stimulation).

In this study, we used a correlational strategy to investigate the functional substrates of the ontogeny of TWA behavior. To record regional brain activity, we applied a modification of the 2-DG method, the 2-Fluoro-deoxyglucose (2-FDG) method (Gonzalez-Lima, 1992; Bock et al., 1997).
We wanted to answer the following general questions:
1) Is there a difference in the functional activity of single brain areas between infant (insufficient TWA learning) and adolescent (sufficient TWA learning) rats?
2) Is there a difference in the functional activity of single brain areas between different training stages (acquisition vs. retrieval)?
3) Is the behavioral output reflected by differences in the correlated functional activity?
4) Is the performance within a group correlated with the functional activity of single brain regions?

1.4.2. Accomplishment of the study

1.4.2.1. Behavioral conditions

Applying the 2-FDG method, it is not possible to follow up developmental time points or to compare baseline activity with different learning stages in single animals. Thus, the establishment of groups is required. Here, we established eight 'behavioral conditions' covering different age and training stages as well as the respective control groups. Infant (P17-P21) and adolescent (P38-P42) rats were either trained in the TWA task for one day (acquisition) or five days (retrieval) or were solely exposed to the shuttle box without TWA training for one day (novelty) or five days (familiarity)[9].
In line with our previous studies (Schäble et al., 2007; Gruss et al., 2010), it was predicted that:

➔ *the TWA performance of the infant rats is significantly worse than that of the adolescent rats.*

[9] for details, see: Materials and Methods (2.5.)

1.4.2.2. Functional activity in single regions

Within all rats, functional activity[10] was measured in 39 different brain areas (cortico-limbic, hippocampal, amygdaloid, striatal, hypothalamic and brain stem areas as well as primary sensory and motor cortex). All these areas have been shown in the past to contribute to TWA learning and performance (Table 3, Fig. 4).

Investigating developmental task-provoked functional activity during very early ages, Gonzalez-Lima and co-workers showed that, if P17 rats are compared to P12 rats, extinction training results in a higher functional activity at P17 in a variety of limbic, basal forebrain and brain stem areas but not in somatosensory and motor regions (Nair and Gonzalez-Lima, 1999; Nair et al., 2001). Sullivan and colleagues showed a specific increase in functional activity in the amygdala in P12 rats compared to P8 when animals were given odor-shock pairings but two other behavioral challenges were not reflected by age differences (Sullivan et al., 2000). From these findings, it was predicted that:

➔ *the functional activity in brain regions involved in association, decision, emotional and reward processes is lower in infant than in adolescent rats (infant < adolescent). In contrast, primary sensory and motor regions as well as regions primarily involved in encoding and storage of information should show identical task-provoked metabolic activities in infant and adolescent rats (infant = adolescents).*

A small number of studies in humans have provided evidence that better performance requires less energy consumption. Thus, that the better the individual task performance was, the less activation was found in the involved brain regions (Brechmann and Scheich, 2005); or the more difficult a task was, the larger was the activated area (Sunaert et al.,

[10] 2-FDG utilization is a measure for 'metabolic activity'. As the metabolic activity is tightly linked to the function of neurons (see 2.6.: Addendum), it is also called 'functional activity'. Thus, the terms are used synonymously. However, in the 'Introduction' and 'Discussion' the term 'functional activity' is preferred, whereas in 'Materials and Methods' and in the 'Results' part the term 'metabolic activity' is used.

2000). Based upon these findings, it was predicted that:

➔ *in the adolescent rats that appropriately acquire and retrieve the TWA task, functional activity should be higher during acquisition than during retrieval ('adolescent acquisition > 'adolescent retrieval'). In the infant rats, this difference should not be observable ('infant acquisition' = 'infant retrieval').*

1.4.2.3. Correlated functional activity

To analyze the functional brain activity in single brain areas is a reasonable starting point. However, the cognitive demands, which are necessary to translate specific environmental challenges into behavioral output, are rather mediated by differential functional networks (Stevens, 2009). Perception and association of sensory stimuli, the generation of a behavioral plan and its transcription into a physical action require the coordinated, fine-tuned interaction of many brain regions (Fig. 4). Thus, within the next step, we compared the correlated functional activity[11] between the eight behavioral conditions.

Only few previous studies have investigated correlated brain activity in a similar way. They showed, e.g., that the younger the rat pups were (P12 vs. P17), the more uncoupled were frontal cortical and limbic regions during a behavioral challenge (Barrett et al., 2003; Nair et al., 1999; Nair et al., 2001). Based upon these findings, it was predicted that:

➔ *age and training increase the correlated functional activity.*

[11] by compiling inter-regional correlations of metabolic activities

1.4.2.4. Correlations of behavior and functional activity

Finally, we wanted to show that *within* the behavioral condition that covers animals, which appropriately learned the TWA task, the functional activity is statistically related to the behavioral performance. Thus, according to the above introduced studies (Brechmann and Scheich, 2005; Sunaert et al., 2000), it was predicted that:

➔ *if the task is sufficiently learned ('adolescent retrieval'), the behavioral performance, i.e. the number of avoidances, and the functional activity will inversely correlate within this group. Such an inverse correlation should be not found in infant rats.*

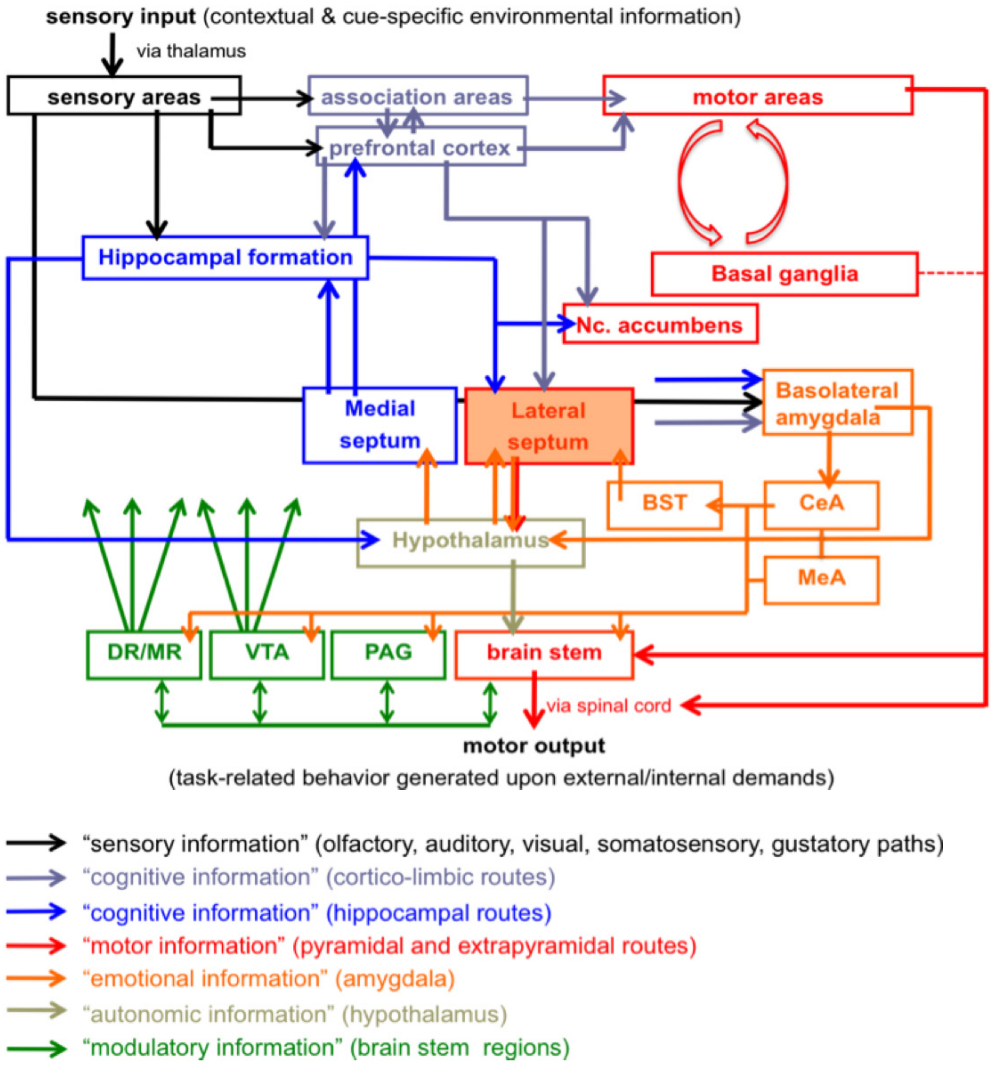


Figure 4: Simplified brain circuitry. Different modes of information take different routes within a number of brain systems (color-coded). Some regions are considered to be transition zones like, e.g., the LS. The widespread projections of the modulatory brain stem regions, i.e. serotonergic raphé and dopaminergic VTA, target a large number of areas throughout the brain.

2. Material and Methods

2.1. Animals

A total of 72 female Wistar rats from the breeding colony at the Leibniz Institute for Neurobiology Magdeburg (strain Schönwalde, Leibniz Institute for Neurobiology) were used for the experiments (67 rats could be finally included in the statistical analysis of metabolic activity due to insufficient brain accumulation of 2-FDG).

Animals were either infant (P17-P21, nine litters) or adolescent (P38-P42, nine litters). As our initial pilot studies consistently revealed a slightly better and less variable TWA performance in females compared to males, all following studies including this one only implemented female Wistar rats (Gruss et al., 2010; Schäble et al., 2007).

All experimental protocols were approved by the ethics committee of the government of Saxony-Anhalt according to the German guidelines for the care and use of animals in laboratory research. The experiments were performed in accordance with the European Communities Council Directive (86/609/EEC). Experiments were generally carried out between 8:00 and 12:00 a.m.

2.2. Housing

Pregnant females were checked for litters daily, and at the day of birth (P0) the litters were standardized to five female and five male pups per dam. The infant rats were kept with their mothers and siblings during the entire experiment, which was finished for them on P21. Animals assigned to the P42 group were removed from their mother and male siblings at P21 and housed litter-wise in cages. All animals were kept in translucent standard laboratory cages Type IV (E. Becker & Co. GmbH, Germany, Castrop-Rauxel) under controlled laboratory conditions (temperature: 21 ± 2 °C; humidity: 55 ± 5%; artificial 12h/12h light/dark cycle) with access to food and water ad libitum. Cage cleaning was done once a week.

2.3. Shuttle-box apparatus

Experiments were conducted in fully automated shuttle-boxes located in ventilated and sound-protected cubicles (TSE Systems GmbH, Germany, Bad Homburg). Infant rats were trained in shuttle-boxes of 30.3 x 23.0 x 20.5 cm size (length x depth x height), equipped with a floor grid of 0.4 cm diameter bars spaced 0.9 cm apart. Adolescent rats were trained in shuttle-boxes of 48.5 x 23.0 x 20.5 cm size with a floor grid of 0.9 cm diameter bars spaced 1.8 cm apart. Shuttle-boxes were bisected by a vertical wall (non-transparent polyvinyl chloride plate), which contained a door allowing the animal to freely move to the opposite compartment. Loudspeakers were placed on top of each compartment. Infrared light beams permanently determined the position of the animal. For system control and data acquisition the shuttle.exe software (TSE Systems GmbH, Germany, Bad Homburg) was used. To minimize odor cues, shuttle-boxes were cleaned with 70% ethanol after each animal. Experiments were generally carried out between 8:00 and 12:00 a.m.

2.4. Trial paradigm and behavioral data

Stable and reliable conditioning was achieved by ***delayed CS-UCS pairing*** (the CS is preceding the UCS and presented in parallel until both switch off) and ***continuous reinforcement***, i.e. every correct reaction was resulting in relief from the unpleasant stimulus. Each training session started with 3 min of habituation allowing the animals to explore the shuttle-box. Afterwards, learning trials were applied as follows (Fig. 5): The CS, a tone of 2.4 kHz frequency and 80 dB loudness, was presented for 5 s maximally. Afterwards, the UCS, a 600 µA foot-shock, was added for a maximal duration of 15 s. If the animal did not move to the other chamber, CS and UCS automatically switched off followed by an inter-trial interval (ITI) of 40 s. Otherwise, the ITI started as soon as the animal left the chamber where CS and UCS were applied.

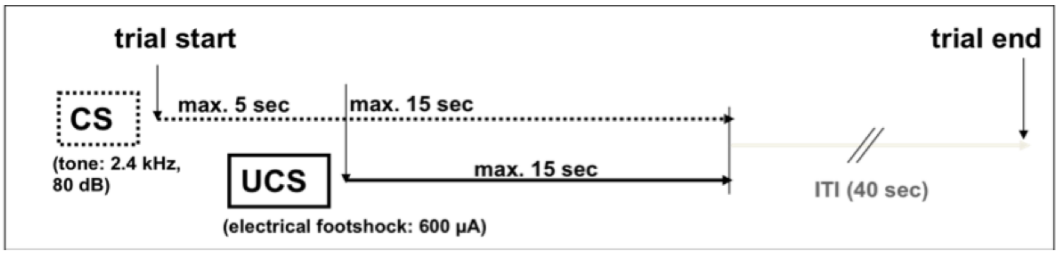


Figure 5: Trial paradigm.

The following parameters were recorded:

1) Habituation parameters (manual recording, i.e. counting of the respective responses by the observer):

- ***number of compartment changes*** (moving back and forth between the two opposite compartments of the shuttle-box, indicator of loco-motor and exploratory activity),
- ***number of exploration responses*** (exploration of the opposite compartment from the door position without moving to the other side),
- ***number of rearing responses*** (complete lifting of the forepaws – freely or attached to a wall, majorly an indicator of explorative activity),
- ***grooming*** (indicator of feeling comfortable and/or displacement activity),
- ***time of inactivity*** (no obvious movement, however, time of inactivity does not mean "freezing"; indicator of loco-motor and exploratory inhibition);

2) Training parameters (automated recording, see chapter 2.3)**:**

- ***number of avoidance reactions*** (compartment change within the 5 sec of CS duration, i.e. prior to UCS onset),
- ***avoidance latency*** (time until the avoidance reaction was completed, measure of velocity),
- ***number of escape reactions*** (compartment change during UCS, i.e. within 15 sec),
- ***escape latency*** (time until the escape response was completed, measure of velocity),
- ***number of failures*** (no compartment change during CS and UCS delivery),
- ***number of inter-trial changes*** (compartment changes during the ITI,

measure of general loco-motor activity).

From these training parameters, the following values were calculated:

- ***cumulative number of avoidances*** (sum of avoidance reactions over 250 trials),
- ***cumulative number of escapes*** (sum of escape reactions over 250 trials),
- ***cumulative number of failures*** (sum of failures over 250 trials),
- ***UCS exposure time*** (sum of escape latencies over the last 50 trials),
- ***cumulative UCS exposure time*** (sum of escape latencies over 250 trials).

Furthermore, the ***number of fecal boli*** (measure of defecation frequency) was counted over the entire habituation and training period. Increasing numbers of fecal boli are an indicator of autonomic responses evoked by stressful or emotionally challenging events (Borelli et al., 2004).

2.5. Experimental groups

36 infant (P17-P21) and 36 adolescent (P38-P42) rats were recruited from nine different litters, respectively. To avoid statistical bias caused by the litter effect (King, 1969), we allotted only one individual of each litter to each condition (i.e. the four infants per litter to the four infant conditions; the four adolescents per litter to the four adolescent conditions). The fifth female sibling was always kept in the home cage of the litter until the end of the experiment.

Novelty and familiarity conditions were established because the context-dependent environmental influences (i.e. shuttle-box without TWA training) also interfere with the functional activity. According to Raichle and Mintun (2006), establishment control conditions, which are at least referable to a physiological baseline is the prerequisite for the reliable interpretation of imaging data (even if they not constitute genuine “zero” or home cage activity).

In the shuttle-box, animals were trained/exposed as follows (Tab. 4):

- ***‘infant acquisition’*** (n=9): 50 consecutive learning trials on P21,
- ***‘infant novelty’*** (n=9): shuttle-box exposure for 40 min without presentation of CS and UCS on P21,

- ***'infant retrieval'*** (n=8): 50 trials on five consecutive days each from P17 to P21,
- ***'infant familiarity'*** (n=7): shuttle-box exposure for 40 min without CS and UCS on five consecutive days from P17-P21,
- ***'adolescent acquisition'*** (n=9): 50 consecutive learning trials on P42,
- ***'adolescent novelty'*** (n=8): shuttle-box exposure for 40 min without presentation of CS and UCS on P42,
- ***'adolescent retrieval'*** (n=9): 50 trials on five consecutive days each from P38 to P42,
- ***'adolescent familiarity'*** (n=8): shuttle-box exposure for 40 min without CS and UCS on five consecutive days from P38 to P42.

Prior to the last training or shuttle-box exposure, rats were intraperitoneally injected with 18 µCi 2-FDG/100g body weight.

Behavioral condition	**Age during TWA training (number of trials)**					**Days of SB exposure (sum of min)**				
infant acquisition	P17 (-)	P18 (-)	P19 (-)	P20 (-)	*P21 (50)					
infant novelty						P17 (-)	P18 (-)	P19 (-)	P20 (-)	*P21 (40')
infant retrieval	P17 (50)	P18 (50)	P19 (50)	P20 (50)	*P21 (50)					
infant familiarity						P17 (40')	P18 (40')	P19 (40')	P20 (40')	*P21 (40')
adolescent acquisition	P38 (-)	P39 (-)	P40 (-)	P41 (-)	*P42 (50)					
adolescent novelty						P38 (-)	P39 (-)	P40 (-)	P41 (-)	*P42 (40')
adolescent retrieval	P38 (50)	P39 (50)	P40 (50)	P41 (50)	*P42 (50)					
adolescent familiarity						P38 (40')	P39 (40')	P40 (40')	P41 (40')	*P42 (40')

Table 4: Experimental conditions. The table illustrates the postnatal days (P) during which the rats were trained. *Prior to the last training or shuttle-box exposure, 18 µCi 2-FDG/100g body weight were applied intraperitoneally. After this last training/exposure, rats were sacrificed.

2.6. The 2-FDG method: functional imaging of the metabolic activity

2-Fluoro-deoxyglucose (2-FDG) is an analogue of glucose, which is transported into cells by glucose transporters, phosphorylated by hexokinase and not further metabolized. Thus, 2-FDG-phosphate accumulates intracellularly providing a quantitative assessment of regional glucose utilization as a measure of functional activity. Currently, it is hypothesized that neurons recruit their energy from direct glucose uptake as well as indirect glucose utilization via uptake of astrocytic lactate (Chih et al., 2001; Magistretti, 2006).

As mentioned above, animals were intraperitoneally injected with 18 µCi 2-FDG (per 100g body weight; American Radiolabeled Chemicals) with a specific activity of 300mCi/mmol at) at P21 or P42 prior to the last training or exposure to the shuttle-box (Tab. 4). Afterwards, the animals were decapitated. The brains were removed from the skulls, rapidly frozen and cryo-sectioned within six days into series of 40 µm thick frontal sections on a cryostat (HM 500-OM, Microm/Thermo Fisher Scientific GmbH, Germany, Walldorf), mounted onto glass slides and rapidly dried (43°C) on a warm plate. Sections were exposed to imaging films (BioMax MR Film, Kodak/Perkin Elmer, USA/CA, Fremont) in film cassettes for two to six hours. Films were developed (G153, Agfa/Fischer-Sehner GmbH, Germany, Berlin), fixed (G354, Agfa/Fischer-Sehner GmbH, Berlin) and dried in a developing machine (Curix, Agfa, Belgium, Mortsel). Images of brain sections were captured with a Camera (FA 87 digital; Grundig GmbH, Germany, Nürnberg) connected to a frame grabber card (Scion Corporation, USA/MD, Frederick) using Scion Image, release Alpha 4.0.3.2 (Scion Corporation, USA/MD, Frederick). Gray values of the background were determined by averaging ten measures per film. For the analysis of metabolic activity, 10 measures (delineation of an area with polygon or ellipse tools by hand) of every area were done in each hemisphere. After subtracting the background value of film from each measurement, the mean absolute optical density was calculated. To compensate for individual differences, it was necessary to calculate relative values (Bock, 1998). Thus, the corpus callosum was defined as the internal reference structure. Within this major fiber tract of the brain,

only a very low signal is detectable. 20 measures of the cc were taken, the background was subtracted and the mean value was calculated. As the 20 measures covered the entire rostro-caudal extent of the brain, every individual brain areas could be referred to the cc value of the individual. To avoid statistical bias, it was checked that the absolute optical density values of the corpus callosum do not differ between the behavioral conditions ($p>0.6$). The relative optical density (rOD) was calculated by dividing the mean value of each brain area by the mean value of corpus callosum (Bock et al., 1997; Konkle and Bielajew, 2004). Thus, metabolic activity (2-FDG utilization) was finally represented by the rOD. To check the correct placement of the regions of interest in the 2-FDG-measure selected sections were counterstained with thionine Nissl stain (Fig. 6).

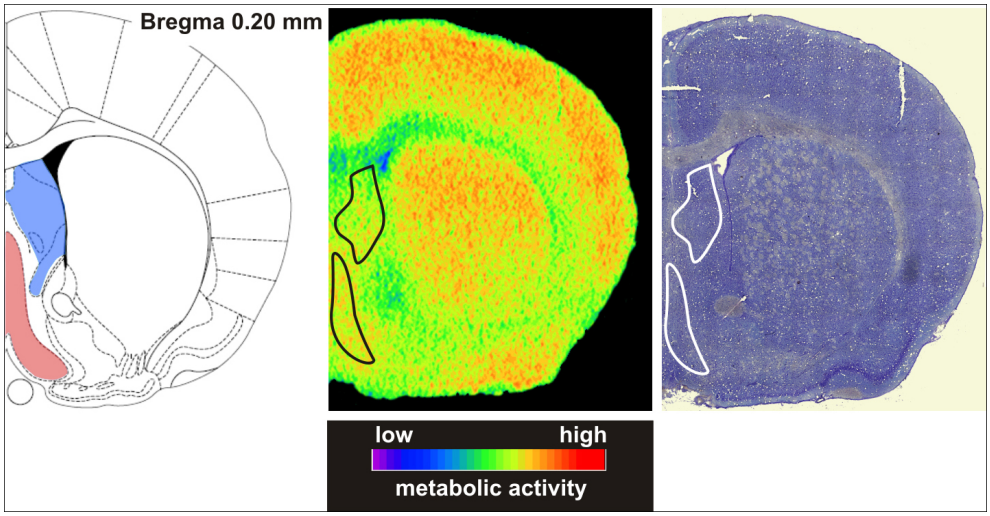


Figure 6: Illustration of the placement of measurements using the example of MS/DB and LS. Left: Selected regions of interest according to Paxinos and Watson (1998); MS/DB – blue, LS – red. Middle: color-coded 2-FDG-labeled section with delineated areas of interest. Right: Nissl-counterstaining for checking the correct placement of the measurements. Below: Range indicator scale of metabolic activity.

Addendum: Physico-chemical background. The 2-FDG method utilizes the coupling between synaptic activity and glucose utilization (neuro-metabolic coupling) for functional imaging based on radioactively labeled glucose molecules. In 1977, Sokoloff and colleagues originally introduced this imaging method, which took advantage of the fact that an experimentally applied glucose analogue is a competitive substrate for the physiological glucose inherent to the organism. Originally, the analogue was 2-deoxyglucose (2DG), a molecule with radiocarbon ^{14}C instead of carbon ^{12}C on position one of the glucose molecule and a split off hydroxy-group (-OH) on position two (Fig. 7).

2-DG (mw = 164,2 Da) is transported via blood-brain barrier and cell membranes similarly to the physiological molecule (mw = 180,2 Da) but after phosphorylation by hexokinase, 2-DG-phosphate cannot be cleaved further on and accumulates in the cells. The period, necessary for this accumulation process leading to detectable

signals is about 40 – 45 min.

The signal that is finally emitted and used to illuminate films or imaging plates is beta radiation (^{14}C ➔ $^{14}N + \beta$). Once the 2-DG is accumulated in the cells, the intensity of β-radiation is proportional to the metabolic activity in terms of oxidative phosphorylation, which is correlated with the electrical activity of cells.

Insertion of a few more modifications into the 2-DG molecule results in generation of 2-FDG. The latter has radiocarbon atoms in all six monosaccharide scaffold positions of the glucose molecule and an additional fluorine atom attached to C2 (Fig. 7). These changes render the 2-FDG molecule (mw = 182,1 Da) much more similar to physiological glucose (faster transport and phosphorylation) and six times more sensitive to detection (Gonzalez-Lima, 1992).

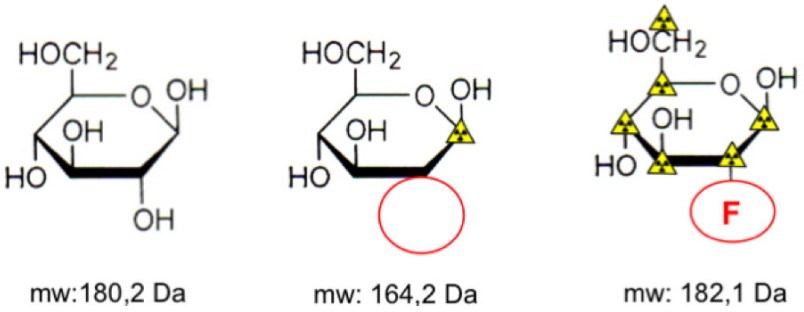


Figure 7: Differences between physiological glucose (left) and derived molecules utilized for imaging methods, i.e. 2-DG (middle) and 2-FDG (right).

Addendum: Biochemical background. Two different hypotheses currently compete in the explanation of glucose utilization by neurons and astrocytes (Fig. 8; Chih et al. 2001).

According to the **conventional hypothesis** neurons utilize glucose **(5)** to produce ATP **(4a,b),** which is consumed by the Na^+-K^+-ATPase restoring the membrane potential after action potential discharge **(1)**. In neurons, glucose is converted to pyruvate **(3a)** that enters the tricyclic acid cycle **(3b)** and mitochondrial oxidative phosphorylation. Finally, decreased cytoplasmic glucose levels lead to increased transport of glucose via the isoform 3 of the glucose transporter (GLUT3; **6a**), which is located in pre- and postsynaptic membranes. Rapid increases in intraneuronal glycolysis lead to increased levels of NADH/NAD^+, H^+ and pyruvate driving the LDH reaction towards lactate production **(7a)**. Simultaneously, in astrocytes, similar glycolytic processes are triggered by high-affinity glutamate and Na^+ uptake from the synaptic cleft **(8)** after neuron discharge. Thereafter, glutamate is converted to glutamine **(9)** and Na^+ is carried outside the cell by Na^+-K^+-ATPase **(2b)**, both processes requiring energy in the form of ATP. In contrast to neurons, glucose is transported into astrocytes by the isoform 1 of glucose transporter (GLUT1; **6b**).

In contrast, the **Astrocyte-neuron lactate shuttle hypothesis (ANLSH)** postulates that primarily high-affinity glutamate/Na+ from the synaptic cleft into astrocytes followed by glutamine synthesis **(9)** and Na^+-K^+-ATPase activation trigger energy consumption. This exclusively activates anaerobic glycolysis **(10)** resulting in lactate production **(7b**). The lactate is transported along its concentration gradient into neurons where it is converted by the LDH-1 into pyruvate **(7a)**. Then, the pyruvate enters the neuronal trycyclic acid cycle and mitochondrial oxidative phosphorylation.

According to the ANLSH, no glycolysis is induced in neurons. This is not entirely feasible. Why should neurons *not* use their glycolysis machinery during functional states if it is regularly used during baseline activity? Brain glucose, which is evenly distributed between the extracellular and intracellular compartments, exceeds the Km for hexokinase (even during neural stimulation when values fall by 20-30%). Moreover, glucose transporters are abundant in synaptic membranes and the neuronal GLUT3 transports glucose seven times faster than the astrocytic GLUT1. Thus, it does not make sense that neuronal function should exclusively depend on astrocytes (Chih et al. 2001).

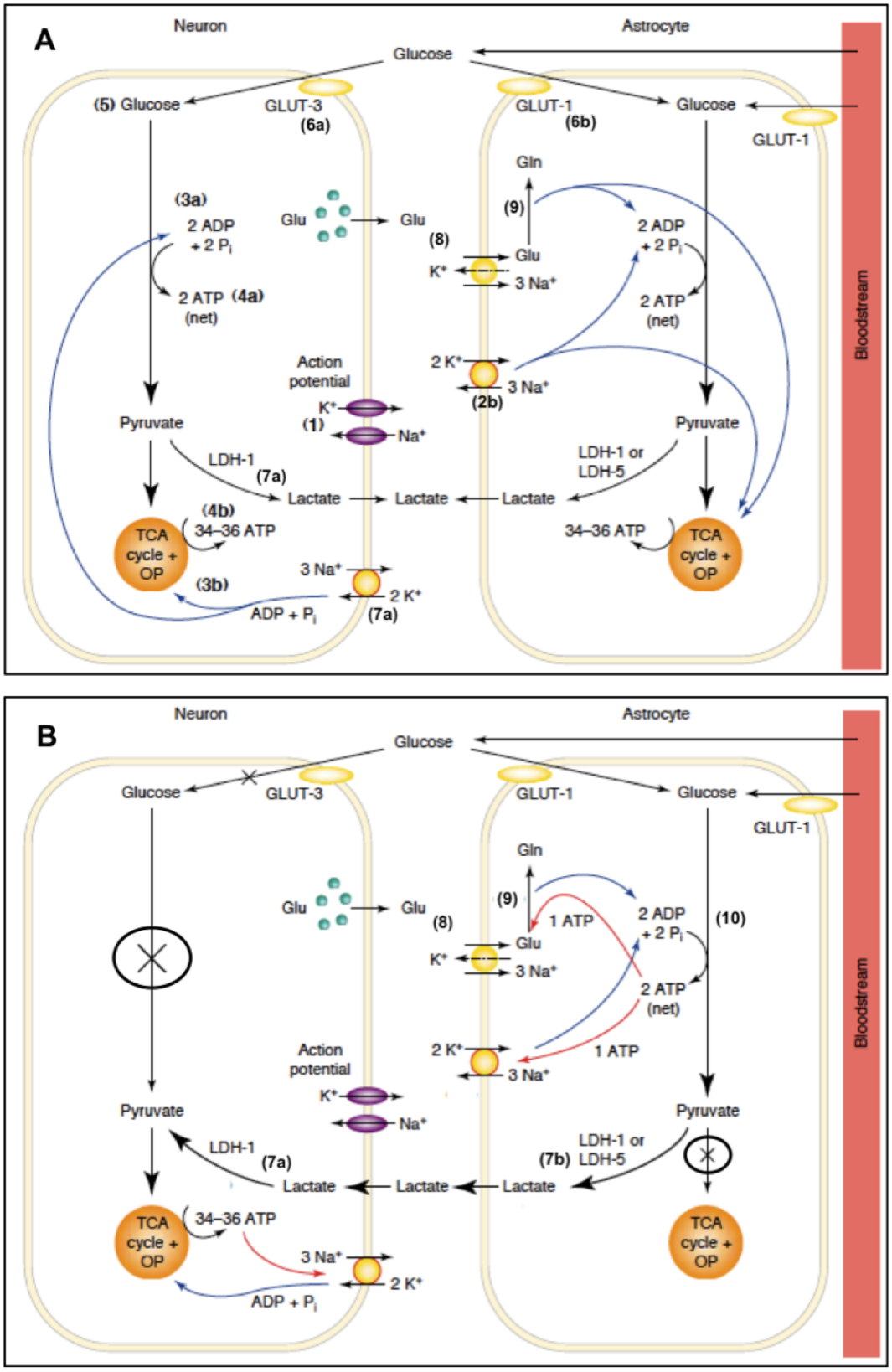


Figure 8: Conventional hypothesis (A) and Astrocyte-neuron lactate shuttle hypothesis (ANLSH; B) of glucose utilization. Explanation, see text. Adapted from: Chih et al. 2001.

2.7. Regions and areas of interest

Altogether, 2-FDG utilization in 39 distinct brain areas was analyzed. The terms “area” and “region” are used to describe different anatomical levels. ***“Area”*** refers to defined cortical areas or subcortical/brain stem nuclei, whereas a ***“region”*** comprises a number of “areas” that are morpho-functionally tightly connected. The areas did anatomically belong to six different regions (Fig. 9; abbreviations see: 5.1.):

1) limbic cortex: VO/LO, IL, PL, Cg1, Cg1/2, RSGb (Σ=6 areas),
2) hippocampal formation: MS/DB, $Hipp_{rost}$, $Hipp_{caud}$, Sub_{rostr}, Sub_{caud} (Σ=5 areas
3) amygdala: $BSTI_{dp}$, CeA, MeA, BL (Σ=4 areas)
4) striatum: ACC, ACS, LS, CP_{dl}, CP_{dm}, CP_{vl}, CP_{vm}, CP_{caud} (Σ=8 areas)
5) hypothalamus: LH, VMH, MM (Σ=3 areas)
6) brain stem/ modulatory areas: VTA, IP, PAG, DR, MR (Σ=5 areas)
7) sensory/motor areas: PIR, M1, S1HL, S1BF, Au1, MG, IC, V1B (Σ=8 areas

As the measurements in these areas and regions of interest were done within a certain rostro-caudal range, the exact stereotactic Bregma coordinates of the measurements are given here according to Paxinos and Watson (1998) in Tab. 5.

Altogether, about 60.000 measurements, i.e. single by-hand delineations of anatomical areas using polygon, ellipse or rectangle options of the Scion Image software, have been performed. This number includes pilot experiments and initial analyses of the animals that did not show sufficient tracer accumulation.

The number of measurements (M)[12] that were included in the statistical analyses of the present study is 54820 (calculated from the numbers of animals [67], regions [39], hemispheres [2], measurements per area [10] and measurements within the reference structure [20]).

[12] **M** = 54940 = (67 x 39 x 10 x 2) + (67 x 20 x 2) = 52260 + 2680

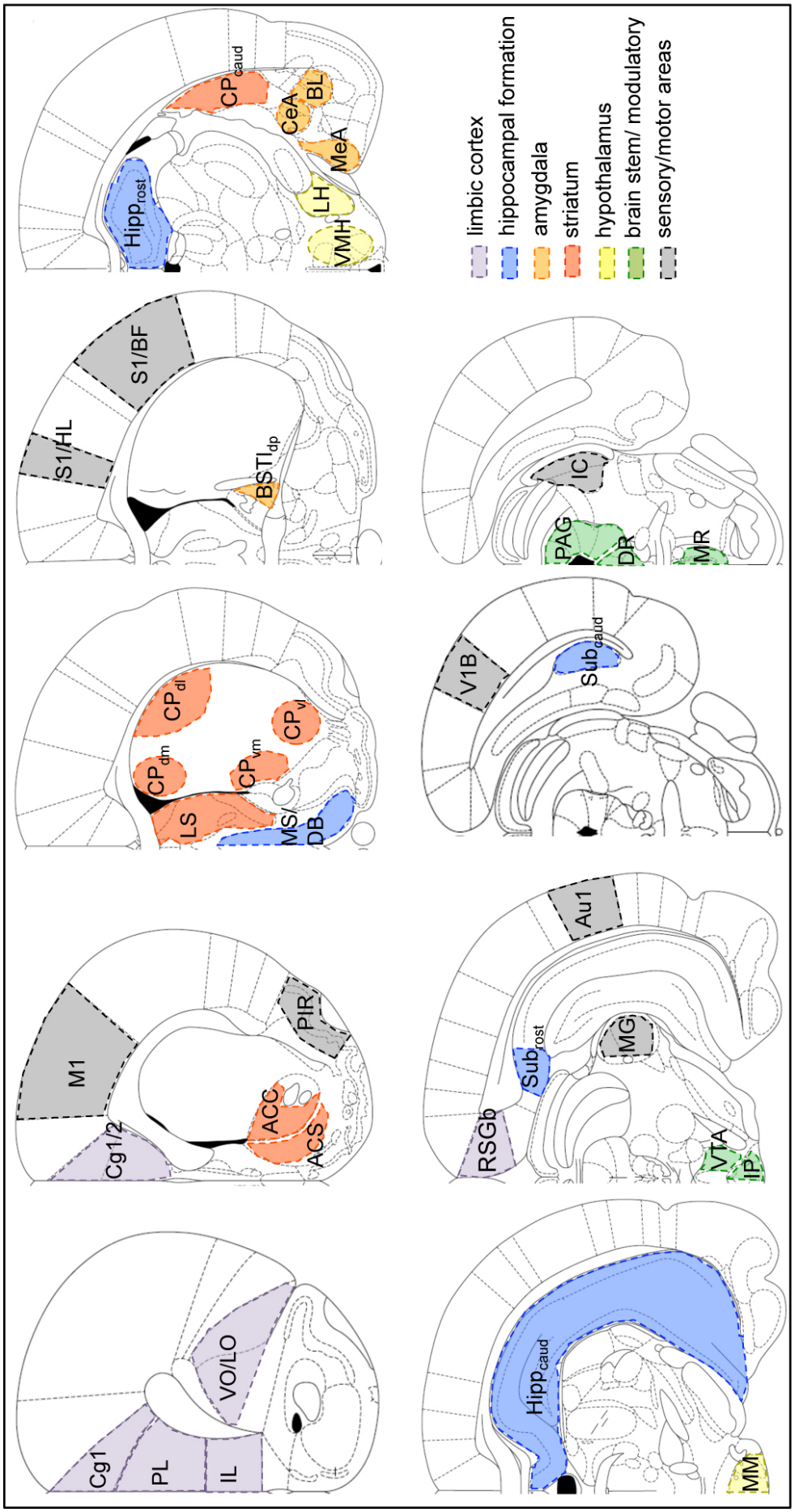


Figure 9: Regions/areas of interest analyzed in the study. Abbreviations: see 5.1.

Region	Area	Coordinates of measurements
limbic cortex	**VO/LO**	Bregma 4.20 to 3.20 mm
limbic cortex	**IL**	Bregma 3.20 to 2.20 mm
limbic cortex	**PL**	Bregma 3.20 to 2.20 mm
limbic cortex	**Cg1**	Bregma 3.20 to 2.20 mm
limbic cortex	**Cg1/2**	Bregma 1.60 to 0.48 mm
limbic cortex	**RSGb**	Bregma -5.60 to -6.72 mm
hippocampal	**MS/DB**	Bregma 0.70 to 0.20 mm
hippocampal	**$Hipp_{rost}$**	Bregma -2.30 to -3.60 mm
hippocampal	**$Hipp_{caud}$**	Bregma -4.80 to -6.04 mm
hippocampal	**Sub_{rost}**	Bregma -5.60 to -6.30 mm
hippocampal	**Sub_{caud}**	Bregma -6.72 to -7.30 mm
amygdaloid	**$BSTI_{dp}$**	Bregma -0.20 to -0.80 mm
amygdaloid	**CeA**	Bregma -1.60 to -2.30 mm
amygdaloid	**MeA**	Bregma -2.12 to -3.30 mm
amygdaloid	**BL**	Bregma -2.30 to -3.30 mm
striatal	**ACC**	Bregma 2.20 to 1.00 mm
striatal	**ACS**	Bregma 2.20 to 1.00 mm
striatal	**LS**	Bregma 1.20 to 0.20 mm
striatal	**CP_{dl}**	Bregma 1.60 to 0.48 mm
striatal	**CP_{dm}**	Bregma 1.60 to 0.48 mm
striatal	**CP_{vl}**	Bregma 1.60 to 0.48 mm
striatal	**CP_{vm}**	Bregma 1.60 to 0.48 mm
striatal	**CP_{caud}**	Bregma -1.88 to -2.80 mm
hypothalamic	**LH**	Bregma -1.80 to -2.56 mm
hypothalamic	**VMH**	Bregma -2.30 to -3.14 mm
hypothalamic	**MM**	Bregma -4.30 to -4.80 mm
brain stem	**VTA**	Bregma -5.80 to -6.30 mm
brain stem	**IP**	Bregma -5.60 to -6.80 mm
brain stem	**PAG**	Bregma -6.80 to 7.80 mm
brain stem	**DR**	Bregma -7.30 to -8.00 mm
brain stem	**MR**	Bregma -7.64 to -8.30 mm
sensory	**PIR**	Bregma 2.20 to 1.00 mm
motor	**M1**	Bregma 1.70 to 0.70 mm
sensory	**S1HL**	Bregma -0.26 to -1.60 mm
sensory	**S1BF**	Bregma -2.56 to -3.60 mm
sensory	**Au1**	Bregma -3.60 to -4.80 mm
sensory	**MG**	Bregma 5.30 to -6.04 mm
sensory	**IC**	Bregma -7.80 to -8.72 mm
sensory	**V1B**	Bregma -6.72 to -7.80 mm

Table 5: Regions/areas analyzed in the study. The areas are summarized as 'regions' to illustrate their morpho-functional similarities. Bregma coordinates are given according to Paxinos and Watson (1998).

2.8. Statistical analyses

Data analysis and diagram compilation were performed with JMP, release 7 (SAS Institute Inc., USA/NC, Cary) and SigmaPlot, release 11.0 (Systat Software Inc., Germany, Erkrath).

Behavioral data were analyzed using either Multivariate analysis of variance (MANOVA) with repeated-measures specification (infant vs. adolescent retrieval groups) or Wilcoxon rank sum test (infant vs. adolescent acquisition and acquisition vs. retrieval on P21/P42; Fig. 14). Second, analyses of cumulative behavioral data were done with all-in-one using Kruskal-Wallis and pair-wise comparisons using Wilcoxon rank sum test (Fig. 16, suppl. Tab. 1).

Metabolic activity, i.e. rOD, was analyzed on three different levels. First, factor analyses, i.e. influence of “condition” and “hemisphere” on the metabolic activity of single areas, were performed using standard least square regressions (suppl. Tab. 2). Post-hoc tests were done by pair-wise comparisons using Tukey’s Honestly Significant Difference (Tukey’s HSD) test (Fig. 17 to 24; suppl. Tab. 2). The statistics software *JMP* simply gives the information whether two groups are significantly different from each other according to the significance level, which is set. This information is encoded by capital letters. Thus, groups (behavioral conditions in our case) that *are not* connected by the same capital letter are statistically different from each other. Vice versa, behavioral conditions, which *are* connected by the same capital letter, are not significantly different from each other. This encoding is used within the box plot diagrams (Fig. 17 to 24) as well as in supplementary Tab. 2. Box plots were compiled as they illustrate not only the mean ± SER but also the distribution (dispersion, skewness and outliers) of non-parametric data. As box plots – essentially the whiskers – are not bijectively defined, a brief description of the box plots compiled in this study is given in Fig. 10.

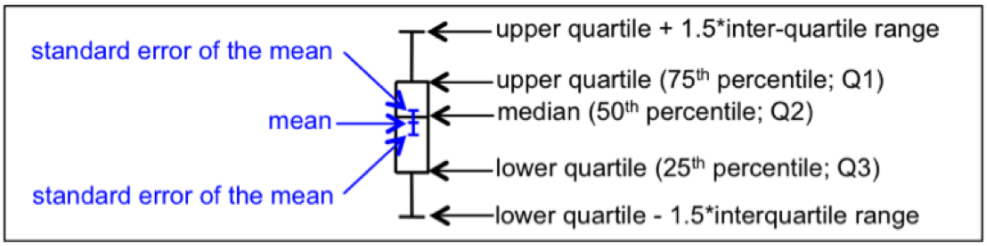


Figure 10: Explanation of box plots compiled in this study.

Second, data exploration and reduction were done by a Principal Component Analysis (PCA) based on a correlation matrix (Tab. 6; for more detailed information see the Addendum below).

Third, with the "cleansed" data set, inter-regional correlations of rODs were calculated for every single behavioral condition indicating positive, negative or no statistical dependency between the metabolic activities of two brain areas ("Correlated functional activity"; Fig. 25). The correlations were calculated using Spearman's rank correlation coefficient, usually called "Spearman's rho (ρ)". The Spearman correlation is a measure of statistical dependencies between two non-parametric variables. It is less sensitive to strong outliers than the Pearson correlation (for parametric data). When the two variables being compared are monotonically related, Spearman correlations of 1 result (-1 indicates significant inverse relationship). As the relationship of the two variables is not linear, Spearman correlations cannot be graphically visualized using linear bivariate fits. Thus, to illustrate the strengths and direction of correlations, mosaic plots were compiled.

Finally, correlation of behavioral and metabolic activity was also done using Spearman's rho (Fig. 26). Significance level (α) was always set 5%.

Addendum: Principal Components Analysis. PCA is an exploratory tool to uncover unknown trends and for finding patterns in high-dimensional data sets, a so-called multivariate analysis. In this context, the terms "variables", "factors" and "conditions" are often used synonymously to describe the number of dimensions in data sets for the observed/measured variable(s). In the present study, the measured variable is the rOD, which varies according to the number of areas measured (39). The latter constitute the different dimensions.

When measuring the rOD of only two different areas, it is easy to plot and visually assess the correlation between them. In contrast, thousands of measurements across different areas have been collected here. Therefore, it becomes impossible to make a visual inspection of the relationship between measurements in such a multi-dimensional data matrix. PCA is a simple data decomposition technique that

reduces the dimensionality of the data set by revealing a small number of independent linear combinations (principal components) of a set of variables that capture as much as possible of the variability within the original data set.

In a PCA a new coordinate system is created from the observed data. The origin of this new coordinate system, which is the grand mean, becomes 0 in the transformed one. The principal components are derived from so-called eigenvalue decomposition of the correlation matrix (decomposition of a covariance matrix or of the unscaled and uncentered data is also possible). In this study, the rODs of x1…x39 form a data cloud in a 39-dimensional space.

The first principal component (first axis) linearly extends through the longest extent of the data cloud; the second component (second axis) is orthogonal to the first axis, extending through the next-longest side of the cloud (Fig. 9). The third component (third axis) is, again, orthogonal to both previous axes. Finally, the vectors of coordinates become uncorrelated ("extracted"). Interpretation of the extracted components depends on the problem.

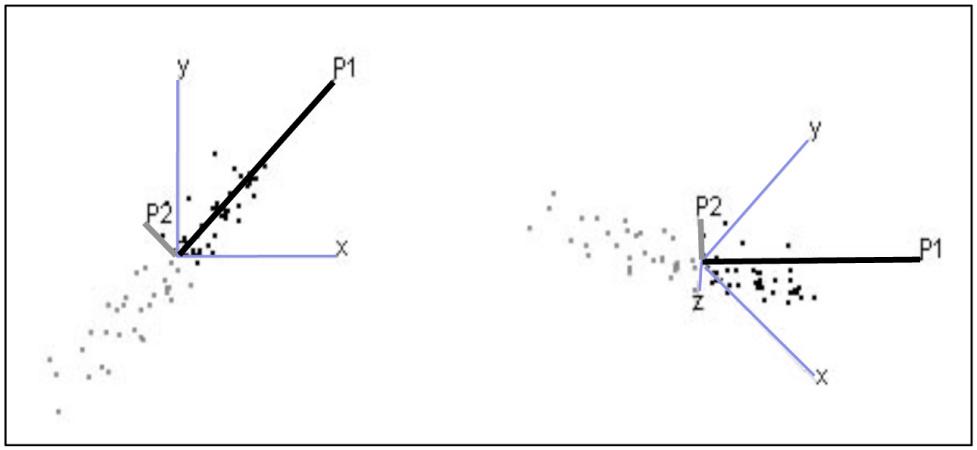


Figure 11: Illustration of two principal components. The scatter plots show two highly correlated variables (black and gray dots). Computation of a linear combination of the two variables that best captures the scatter of the points reveals a principal component (P1) line as shown on the left. Rotating and reflecting the plot so that P1 is the variable on the main axis, gives the best one-dimensional approximation of a two-dimensional cloud of points (right). The second principal component, P2, describes the remaining variation. Similarly, it is possible to plot the best three-dimensional view of higher dimensional data by placing their first three principal components as the spinning axes and examining the plot. Adapted from Statistics and graphics user guide for JMP 7 (2007).

For further understanding, a brief description of the PCA data output table compiled by the software used is given in the next paragraph.

In the initial principal components report (Fig. 10, left table), 'number' enumerates the number of principal components extracted, which are 30 in this study. In contrast to covariance-based PCAs, the number of principal components can be smaller than the number of dimensions in correlation-based PCAs. The 'eigenvalue' corresponds to each principal component in order from largest to smallest. The eigenvalues represent a partition of the total variation in the multivariate sample. They sum to the number of variables (39) when the principal components analysis is based on a correlation matrix. 'Percent' lists each eigenvalue as a percent of the total variance. In our data set, the first principal component accounts for 65.875% of the variation in the sample. The Pareto plot to the right of this column shows the same information in graphical form. 'Cum Percent' displays the cumulative percentage of variation represented by the eigenvalues. In our data set, the first three principal components account for 81.32% of the entire variation. The 'scree plot' shows the eigenvalues, i.e. the fraction of total variance represented by each single component.

According to the eigenvalue, the investigator determines the number of relevant

components. In biological samples like ours, principal components with eigenvalues > 1 were chosen (Korz, 2006; Ohl et al., 2003; Veening et al., 2009). These usually account for a variation between 70 and 90% and, thus cover the lion's share of variation. A number of factors – areas in our study – contribute to each single principal component. For a better interpretation of the principal components a data rotation is now performed resulting in better alignment of the factors within one principal component. We specified the rotation type using orthogonal Varimax rotation, which is the most frequently used rotation matrix. Orthogonal rotation keeps the factors constituting the principal components independent from each other, which is a basic principle of this type of analyses. The output table (Fig. 10, right table) shows the so-called loadings for the single areas (factors) within the rotated factor patterns for the three principal components. The interpretation of this is done as follows: 0 = no loading/no contribution to this principal component; 1 = highest possible loading/maximum relevance for this principal component, -1 = highest negative loading/maximum inverse relevance. We considered areas with loadings ≥ 0.7 relevant for the constitution of the principle component (Tab. 6; Savonenko et al., 1999b; Veening et al., 2009). Loadings < 0.7 ≥ 0.5 were also included in the table to illustrate the tendencies. Loadings < 0.5 were not shown.

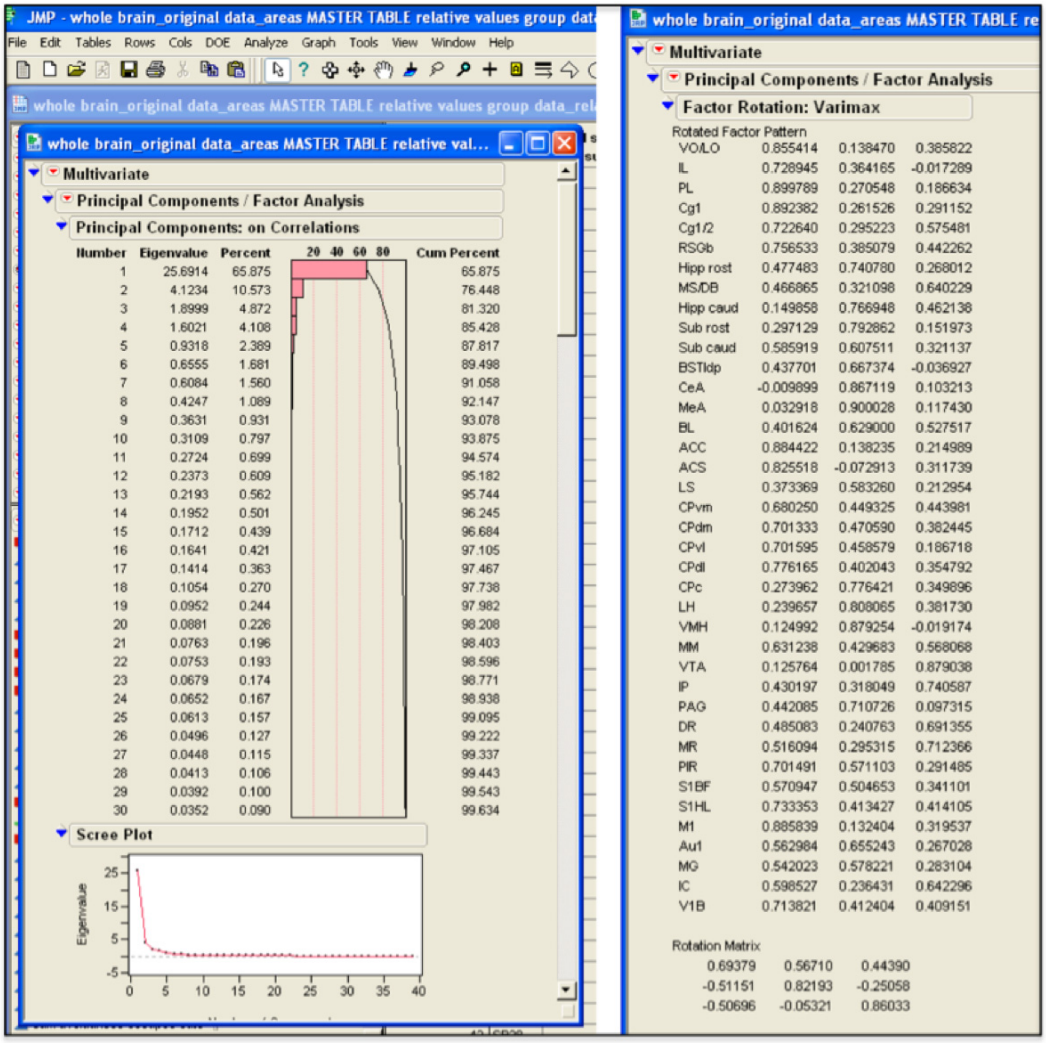


Figure 12: JMP output table of PCA data. Initial principal components report, scree plot and rotated factor pattern. Description see running text: *"Addendum: Principal Components Analysis."*

3. Results

3.1. Behavioral analyses

3.1.1. Habituation parameters

The habituation parameters are summarized in Fig. 13. They were plotted to give a general impression but not for the purpose of statistical analysis[13].

The most important observation was that on training day two, ‘adolescent retrieval’ rats displayed a strong decrease in the three parameters that reflect exploration activity (numbers of compartment changes, exploration responses and rearing responses). This was followed by a re-increase of these three parameters on the third training day.

Inversely, the time of inactivity showed a sharp increase on training day two followed by a re-decrease on day three. Thus, in the ‘adolescent retrieval’ rats, there seemed to be a prominent inhibition of explorative behavior reflected by decreased loco-motor activity after the first TWA training, which dissipated after the second training.

This pronounced inhibition of exploration behavior on day two was not found in the ‘infant retrieval’ condition. Besides, the infant rats – and especially those of the ‘infant retrieval’ group – displayed a very pronounced grooming behavior.

The rats that experienced the shuttle-box environment for five consecutive days without training, i.e. ‘infant familiarity’ and ‘adolescent familiarity’ animals, did not show an inhibition of explorative and loco-motor behavior on the second day of habituation.

In the rats experiencing their first (and last) training on P21 or P42, respectively, it was obvious that the explorative and loco-motor activity was lower in the infants compared to the adolescents. In contrast, the time spent grooming was higher in infants than rats and there were no differences in the time of inactivity (Fig. 13).

[13] This would add a huge data set, which is of minor relevance for the analysis

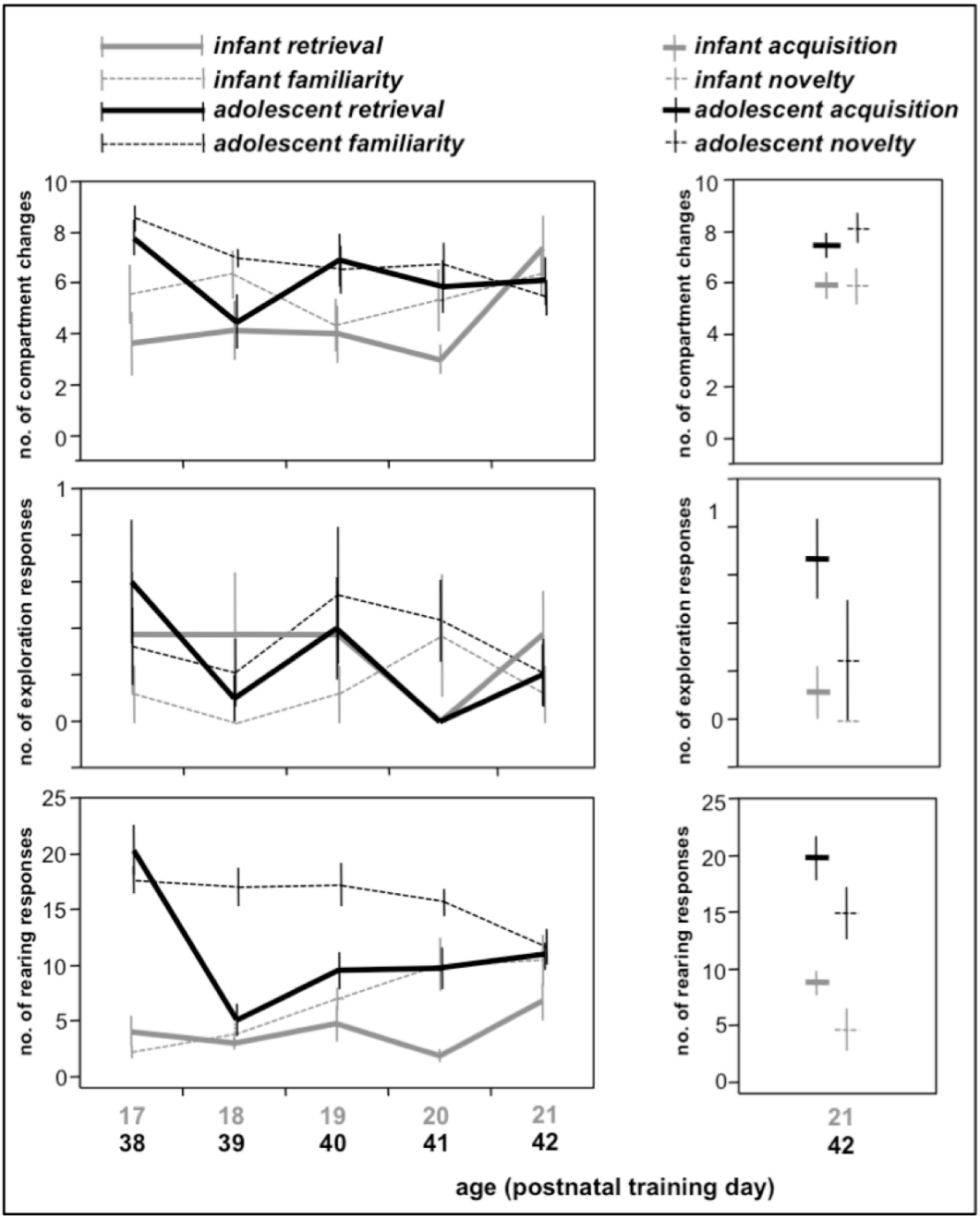


Figure 13: Habituation data. Graphs illustrate the mean ± SEM. Generally, there is a high variance, which is typical for behavioral data. The left column illustrates the four behavioral conditions where animals were trained or exposed to the shuttle-box for five subsequent days. The right column illustrates the four behavioral conditions where animals were trained or exposed to the shuttle-box for one day only.

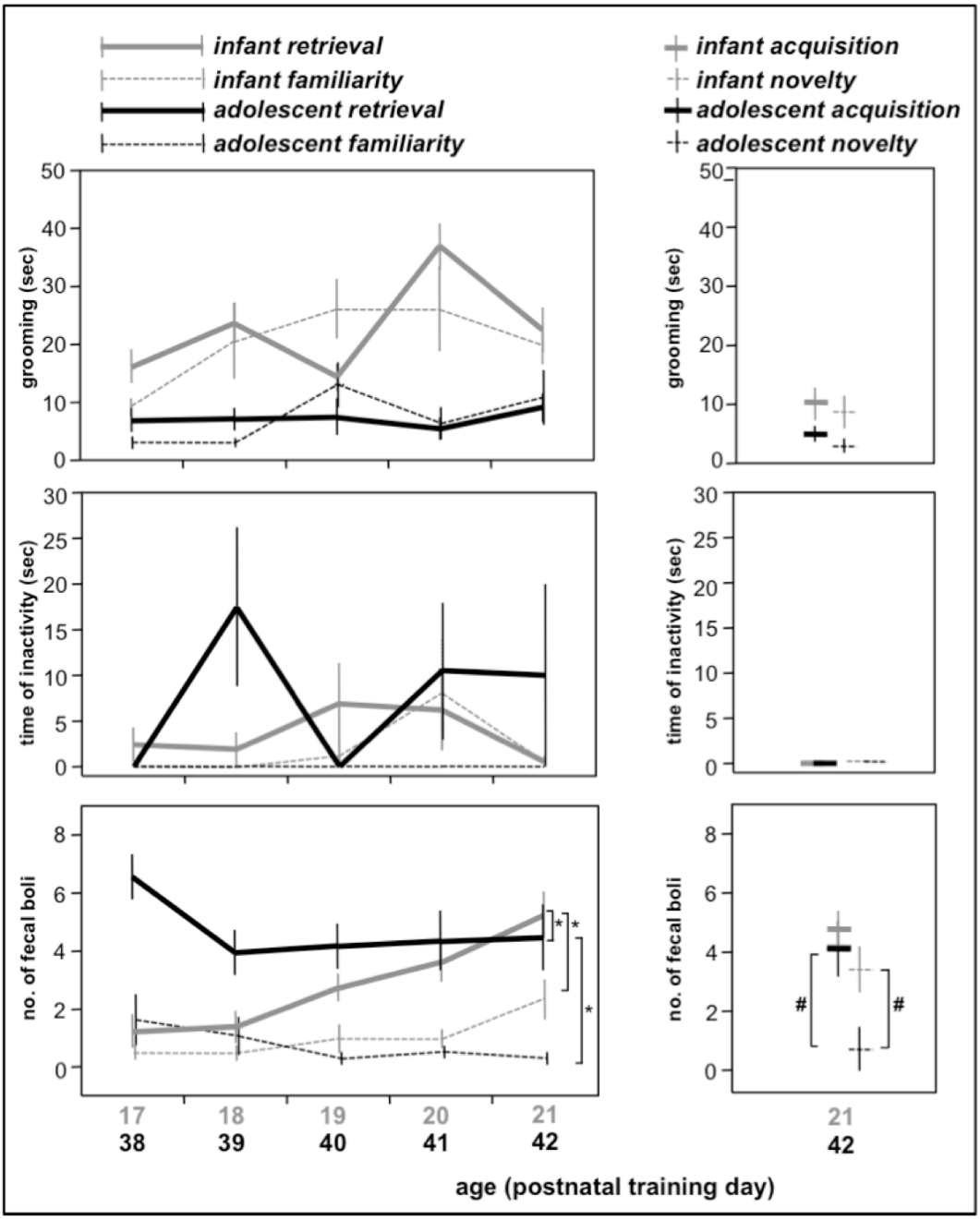


Figure 13 continued: Habituation data. Legend text: see part one of Fig. 13. The number of fecal boli (sum of all boli excreted during habituation and traininig/exposure) is plotted in the bottom row. *Significant differences between conditions covering five days of training/exposure revealed by repeated-measures ANOVA. #Significant differences between conditions covering one day of training/ exposure revealed by pair-wise Wilcoxon rank sum test. $P < 0.05$.

3.1.2. Fecal boli

The number of fecal boli excreted during habituation and training, is plotted in the bottom row of Fig. 13. For the conditions covering five 33training/exposure days, repeated measures ANOVA revealed significant differences 1) between 'infant retrieval' and 'adolescent retrieval'

($F_{(1,15)}$=6.98, p=0.02), 2) between ‘infant retrieval’ and ‘infant familiarity’ ($F_{(1,13)}$=8.44, p=0.0009) as well as 3) between ‘adolescent retrieval’ and ‘adolescent familiarity’ ($F_{(1,15)}$=28.98, p=0.0001).

For the conditions covering one day of training/exposure, pair-wise comparisons revealed significant differences between ‘infant novelty’ and ‘adolescent novelty’ ($F_{(1,17)}$=8.45, p=0.037) as well as between ‘adolescent acquisition’ and ‘adolescent novelty’ ($F_{(1,15)}$=5.28, p=0.02).

3.1.3. Training parameters

3.1.3.1. Time course analysis between the retrieval conditions

For the rats exposed to five days of consecutive training, repeated measures ANOVA revealed a lower number of avoidance reactions ($F_{(1,15)}$=5.80, p=0.03) during ‘infant retrieval’ compared to ‘adolescent retrieval’ but no difference in the avoidance latency (Fig. 14). The number of escape reactions did not differ between ‘infant retrieval’ and ‘adolescent retrieval’. In contrast, the escape latency was significantly higher during ‘infant retrieval’ compared to ‘adolescent retrieval’ ($F_{(1,15)}$=13.00, p=0.003). The number of failures was nearly significantly higher during ‘infant retrieval’ compared to ‘adolescent retrieval’ ($F_{(1,15)}$=4.32, p=0.05). Finally, repeated measures ANOVA revealed a tendency towards significance in the number of inter-trial changes ($F_{(1,15)}$=3.38, p=0.08) with the ‘infant retrieval’ rats displaying a higher number of inter-trial changes than the ‘adolescent retrieval’ animals.

3.1.3.2. Time point analysis between the acquisition conditions

Pair-wise comparisons using Wilcoxon rank sum test revealed a strong tendency towards significance in the number of avoidances ($Chi^2_{(n=18)}$=3.83, p=0.0504) between ‘infant acquisition’ – displaying a lower number – and ‘adolescent acquisition’ – displaying a higher number of avoidances. No significant difference in the numbers of escapes, failures and inter-trial changes as well as in avoidance and escape latency was revealed between ‘infant acquisition’ and ‘adolescent acquisition’ (Fig. 14).

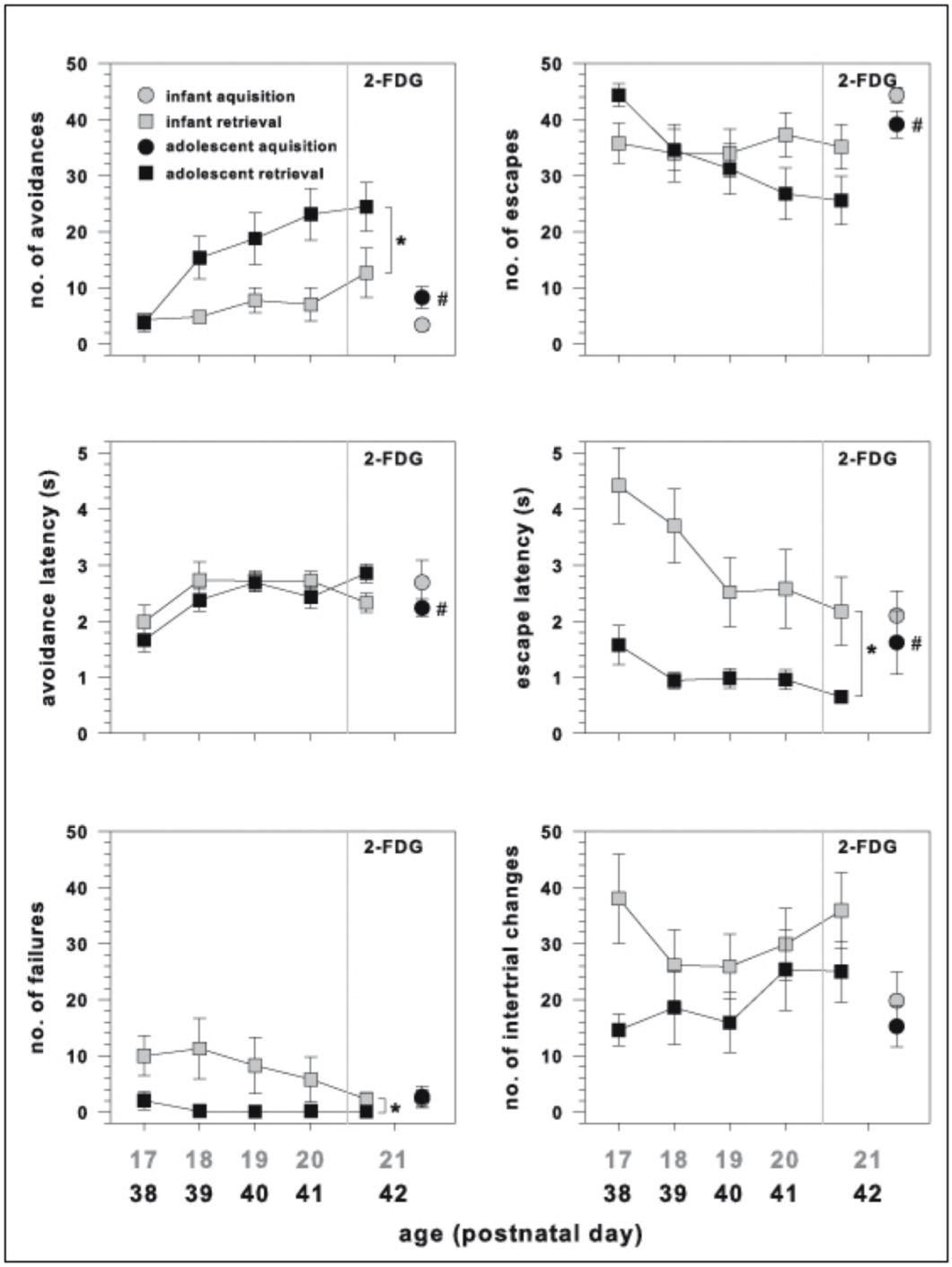


Figure 14: Summary of behavioral parameters. These graphs illustrate the development of performance over days (retrieval groups, squares) compared to one day of training (acquisition groups, circles). *Significant differences between 'infant retrieval' and 'adolescent retrieval' (repeated measures ANOVA; $p < 0.05$). #Significant difference between 'infant acquisition' and 'infant retrieval' on P21 and between 'adolescent acquisition' and 'adolescent retrieval' on P42 (Wilcoxon rank sum test; $p < 0.05$). Adopted from Riedel et al. (2010).

3.1.3.3. Time point analysis between acquisition and retrieval on the last training (P21 or P42, resp.)

Finally, the behavioral parameters measured directly during 2-FDG accumulation were compared pair-wise using Wilcoxon rank sum test. Thus, the fifth (and last) training day for the retrieval groups was compared to the first (and last) training day for the acquisition groups.

For the infant rats, this day was P21, for the adolescent rats it was P42 (Fig. 14, hash signs).

On P21**,** infant rats displayed a non-significantly higher number of avoidances ($Chi^2_{(n=17)}$=3.23, p=0.07) during their fifth day of training ('infant retrieval') compared to their first day of training ('infant acquisition') implying that they did not develop a sufficient avoidance strategy. Similarly, on P21, infant rats displayed non-significantly higher numbers of escapes ($Chi^2_{(n=17)}$=3.36, p=0.07) and inter-trial changes ($Chi^2_{(n=17)}$=3.35, p=0.07) during their fifth day of training ('infant retrieval) compared to their first day of training ('infant acquisition'). On P21, avoidance latency, escape latency and the number of failures did not differ between 'infant retrieval' and 'infant acquisition'.

On P42, adolescent rats displayed a significantly higher number of avoidances ($Chi^2_{(n=18)}$=7.04, p=0.008) and a significantly lower number of escapes ($Chi^2_{(n=18)}$=5.08, p=0.02) during their fifth day of training ('adolescent retrieval) compared to their first day of training ('adolescent acquisition') indicating that they developed a sufficient avoidance strategy. Furthermore, on P42, the avoidance latency ($Chi^2_{(n=18)}$=5.07, p=0.02) was significantly higher and the escape latency ($Chi^2_{(n=18)}$=5.89, p=0.015) was significantly lower during the fifth day of training ('adolescent retrieval) compared to the first day of training ('adolescent acquisition'). The adolescent rats did not emit any failure during the fifth day of training ('adolescent retrieval) compared to the first day of training ('adolescent acquisition'; $Chi^2_{(n=18)}$=3.35, p=0.07). Finally, on P42, the number of inter-trial changes did not differ between 'adolescent retrieval' and 'adolescent acquisition'.

Addendum: Individual learning profiles. To illustrate the differences between 'infant retrieval' and 'adolescent retrieval', the behavioral parameters of two individual animals have been discretely plotted over all 250 trials (Fig. 15).

Avoidance reactions are almost absent between the first 50 trials in both infant and adolescent rats. However, whereas the adolescent animal primarily escapes during the first training day, the infant animal displays also a number of failures. During the course of trial 50 to 250 (2nd to 5th training day), avoidance reactions are singular and separate but regular events in the infant rat, whereas they become numerous and almost coherent in the adolescent animal.

After a slow decrease occurring during the initial 100 trials – the escape latency becomes stable in both infant (longer latencies) and adolescent (shorter latencies) rats. Finally, it is appreciable that the motor activity (inter-trial changes) is different: the infant rat exhibits many trials with zero inter-trial changes throughout the 250 trials, which are regularly interrupted by trials with a certain no. of inter-trial changes. In the adolescent rat, there is an increase in the no. of inter-trial changes over time.

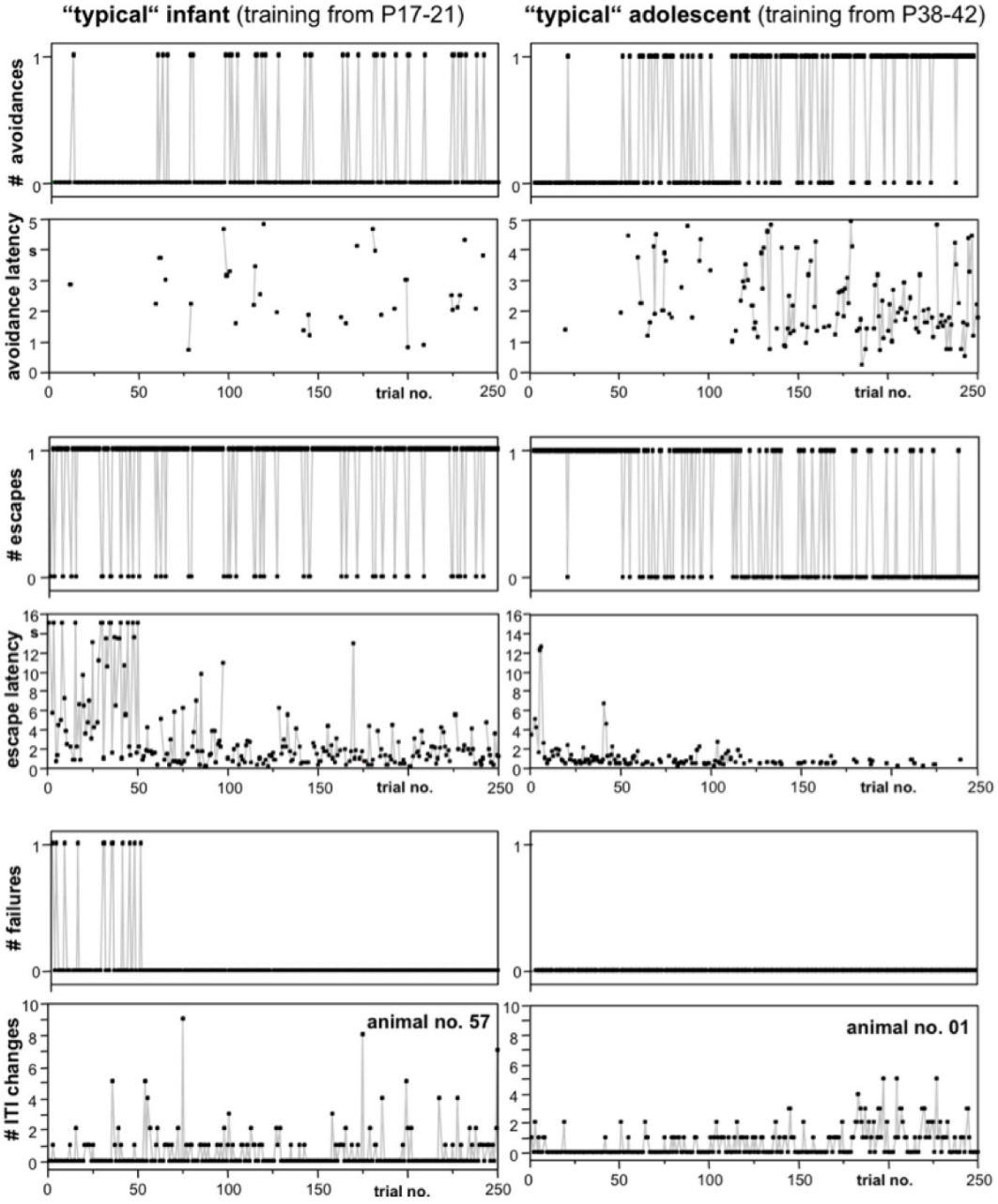


Figure 15: Illustration of the individual performance of an infant (left) and an adolescent (right) rat. Description see running text: *"Addendum: Individual learning profiles."*

3.1.3.4. Cumulative analysis of behavioral parameters

Cumulative analyses allow the concomitant statistical examination of the behavioral parameters otherwise separately evaluated by time course and time point analyses. Thus, although the cumulative analysis basically not reveals new or different results, it completes the general data analyses providing a complete overview of the data, which allows consideration of the data from a different angle (Fig. 16).

On the training day during which 2FDG accumulation was mapped, i.e. P21 for 'infant acquisition' and 'infant retrieval' P42 for 'adolescent acquisition' and 'adolescent retrieval' (black histogram bars in Fig. 16), all-in-one comparisons of the four training conditions using Kruskal-Wallis rank sum test revealed a significant difference for the number of avoidances ($Chi^2_{(3,35)}$=14.55, p=0.002) and the UCS exposure time ($Chi^2_{(3,35)}$=15.46, p=0.0015) but not for the numbers of escapes ($Chi^2_{(3,35)}$=11.77, p=0.082) and failures ($Chi^2_{(3,35)}$=4.66, p=0.15).

Thereupon, post-hoc pair-wise comparisons using Wilcoxon rank sum test have been performed on P21/P42 revealing a significant difference for the number of avoidances between 'infant acquisition' and 'adolescent acquisition' ($Chi^2_{(n=17)}$=3.83, p=0.050), 'adolescent acquisition' and 'adolescent retrieval' ($Chi^2_{(n=17)}$=7.04, p=0.008) and a tendency towards significance between 'infant retrieval' and 'adolescent retrieval' ($Chi^2_{(n=17)}$=3.53, p=0.060). In addition, post-hoc pair-wise comparisons revealed a significant difference in the number of escapes between 'adolescent acquisition' and 'adolescent retrieval' ($Chi^2_{(n=17)}$=5.08, p=0.024), and in the UCS exposure time between 'adolescent acquisition' and 'adolescent retrieval' $Chi^2_{(n=17)}$=8.75, p=0.031) and between 'infant retrieval' and 'adolescent retrieval' ($Chi^2_{(n=17)}$=5.333, p=0.021).

The cumulative parameters over five training days, i.e. 'infant retrieval' vs. 'adolescent retrieval' were also analyzed pair-wise using Wilcoxon rank sum tests (grey histogram bars in Fig. 16). It was revealed that the cumulative number of avoidances ($Chi^2_{(n=17)}$=4.904, p=0.027) was significantly lower in the 'infant retrieval' compared to 'adolescent retrieval' rats. In contrast, the cumulative number of failures ($Chi^2_{(n=17)}$=5.151, p=0.023) and the cumulative UCS exposure time ($Chi^2_{(n=17)}$=8.333,

p=0.004) were significantly higher in 'infant retrieval' compared to 'adolescent retrieval' rats. The cumulative number of escapes did not differ ($Chi^2_{(n=17)}$=0.593, *p*=0.441). An overview over all pair-wise comparisons performed is given in supplementary table 1 (chapter 7.2.; cave: non-sense comparisons have not been performed).

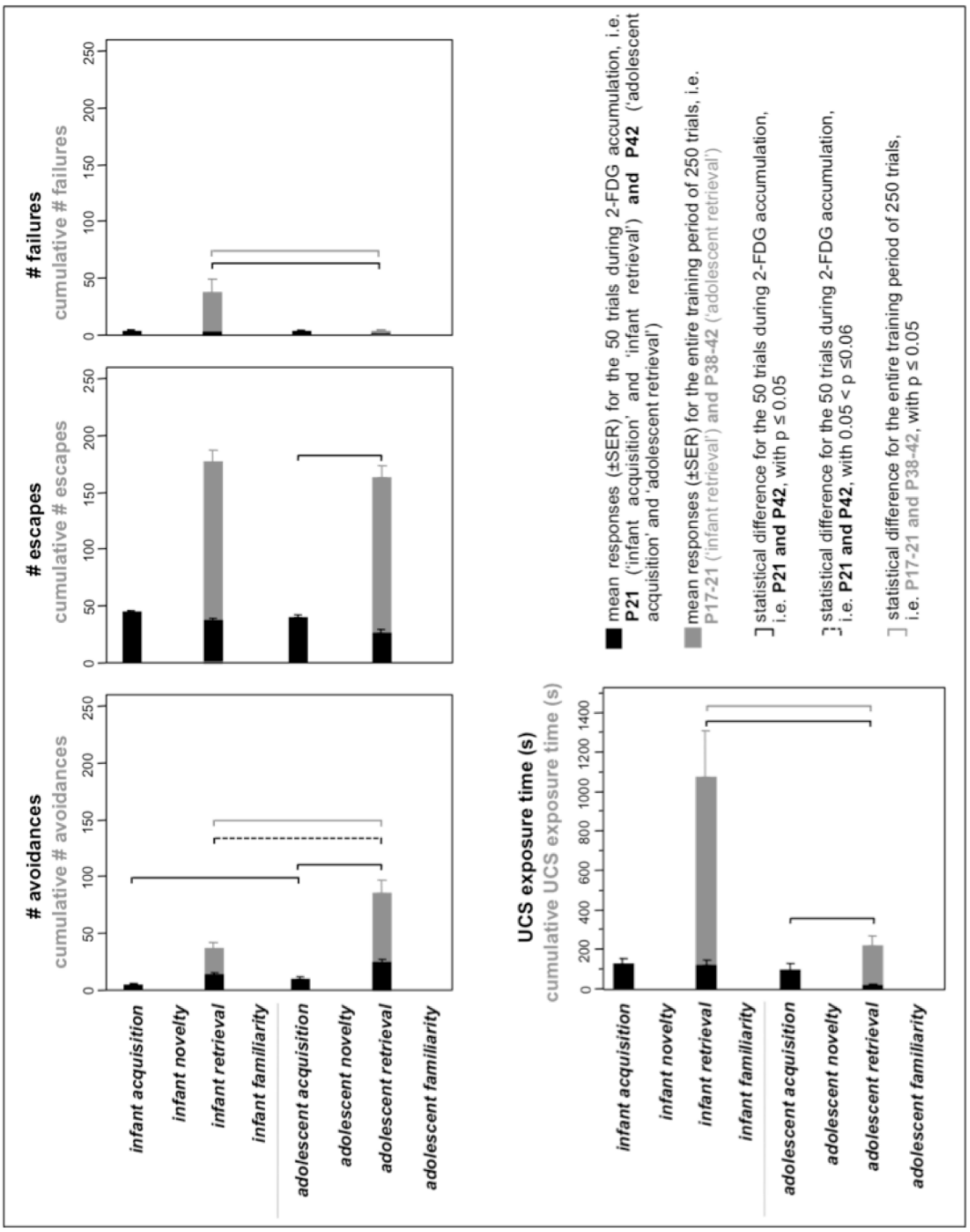


Figure 16: Comparison of cumulative behavioral parameters. The colours of bars and brackets are explained in the legend. Only relevant significances are indicated. See also: supplementary table 1 (chapter 7.2).

3.2. 2-FDG measurements

3.2.1. Influence of 'condition' and 'hemisphere' on the metabolic activity

3.2.1.1. 'Overall metabolic activity'

For the 'overall metabolic activity'[14] comparison, the rODs measurements of all 39 regions have been pooled and averaged (simple arithmetic mean) for each condition. Factor analysis revealed a significant influence of 'condition' on the metabolic activity ($p<0.0001$), whereas 'hemisphere' had no influence ($p=0.645$).

The 'overall metabolic activity' decreases the following, descending order: 'adolescent acquisition' > 'adolescent novelty' > 'adolescent familiarity' > 'adolescent retrieval' > 'infant acquisition' > 'infant familiarity' > 'infant novelty' > 'infant retrieval' (for mean rOD and SEM values, see suppl. Tab. 2).

Post-hoc pair-wise comparisons revealed a number of significant differences (Fig. 17). Most importantly, the adolescent rats, in general display a higher 'overall metabolic activity' than the infant rats.

Among the adolescent rats, 'adolescent acquisition' and 'adolescent novelty' are significantly higher activated than 'adolescent retrieval' but similar to 'adolescent familiarity'. Among the infant rats, 'infant acquisition' is significantly higher activated than 'infant novelty' and 'infant retrieval' but similar to 'infant familiarity'.

[14] the term 'overall metabolic activity' was used to distinguish the "pooled" activity from 'whole-brain activity', which is used as a reference parameter in a number of 2FDG-studies (e.g. Nair and Gonzalez-Lima, 1999).

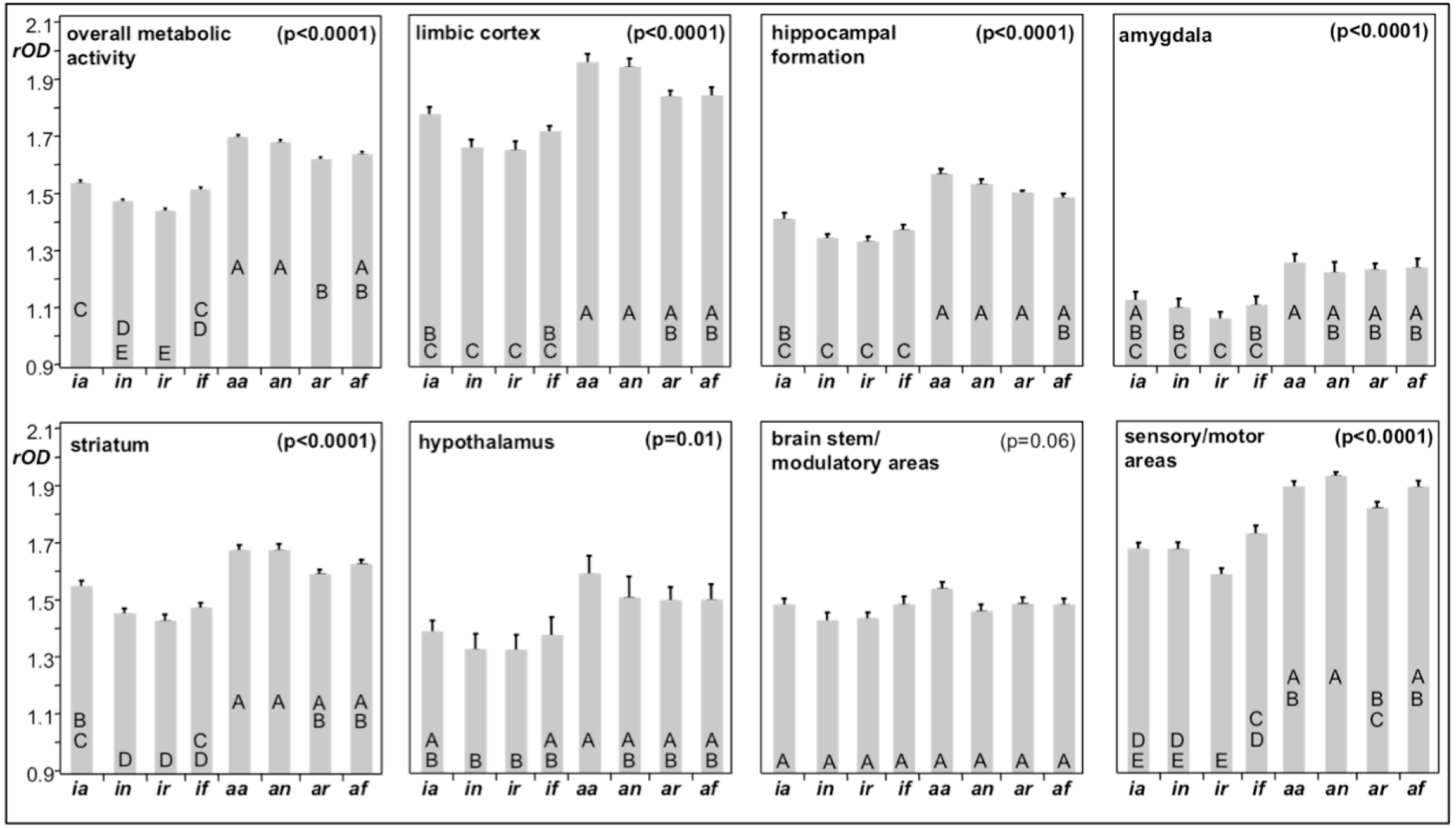


Figure 17: Overall metabolic activity (upper left) and regional metabolic activity. P-values in the diagrams indicate the influence of 'condition' revealed by factor analysis. The pair-wise comparison results revealed by post-hoc Tukey's HSD are indicated with capital letters superimposed on the bars of the histograms: the same letter indicates no significant difference between two groups; different letters indicate a significant difference between two groups. Abbreviations: see 5.2.

3.2.1.2. Regional metabolic activity

At the regional level – i.e. when all rOD measurements of the areas subsumed under a certain region were pooled – the factor 'condition' had a significant influence on the metabolic activity in limbic cortical, hippocampal, amygdaloid, striatal, hypothalamic and sensory/motor ($p<0.0001$) but not in brain stem regions ($p=0.06$; Fig. 17, suppl. Tab. 2). Again, factor 'hemisphere' did not have any influence on the metabolic activity in different regions.

In all regions, the general pattern of metabolic activity was very similar to the 'overall brain activity' (Fig. 17). Post-hoc comparisons revealed that the adolescent rats majorly displayed a higher metabolic activity than the infant rats in most regions (except 'brain stem').

Moreover, there was a higher activity during acquisition than during retrieval in both, infant and adolescent rats; but this difference was only significant in the striatum for 'infant acquisition' vs. 'infant retrieval' at the regional level.

3.2.1.3. Metabolic activity in single brain areas

There was a significant influence of the factor 'condition' in most but not all brain areas (suppl. Tab. 2). Thus, within the ***limbic cortex***, factor 'condition' had significant influence on the metabolic activity of all brain areas ($p \leq 0.019$) except Cg1/2 ($p = 0.133$). Within the ***hippocampal formation***, factor 'condition' had significant influence on the metabolic activity of all brain areas ($p \leq 0.0015$) except MS/DB ($p = 0.0645$). Within the ***amygdala*** ($p \leq 0.001$), ***striatum*** ($p < 0.025$) and ***hypothalamus*** ($p < 0.0013$), factor 'condition' had significant influence on the metabolic activity of all brain areas. Within the ***brain stem***, factor 'condition' had significant influence on the metabolic activity of the VTA ($p = 0.0002$) and PAG ($p < 0.0001$) but not on the metabolic activity of the IP ($p = 0.452$), DR ($p = 0.441$) and MR ($p = 0.618$). Within the ***sensory areas and the primary motor cortex***, factor 'condition' had significant influence on metabolic activity of all areas ($p \leq 0.0058$) except IC ($p = 0.288$). Factor 'hemisphere' did not have a significant influence on the metabolic activity of any brain area investigated ($0.119 \leq p \leq 0.998$), which is in line with the

results of others analyzing 2-FDG utilization bilaterally (Caldecott-Hazard et al, 1988; Soncrant et al., 1986).

Although the all-in-one comparisons frequently revealed statistical differences for the single brain areas, post-hoc comparisons revealed only a number of significant differences (suppl. Tab. 2).

Below, the comparisons of some selected brain areas (Tab. 3) are illustrated and briefly described. The complete collection of data is given in supplementary table 2.

Medial septum/Diagonal Band of Broca & Lateral septum. In the MS/DB, factor 'condition' did only show a tendency towards a significant influence on the metabolic activity. In contrast, factor 'condition' had a significant influence on the metabolic activity of the LS (Fig. 18).

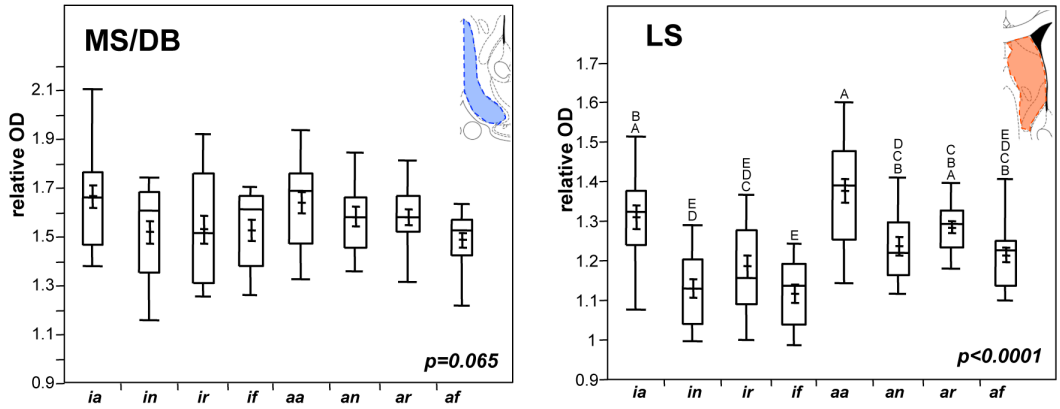


Figure 18: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the MS/DB and LS ($p<0.05$).

In the MS, pair-wise comparisons revealed that all eight behavioral conditions showed a similar 2-FDG utilization (Fig. 18).

In the LS, the adolescent rats displayed a significantly higher metabolic activity than the infant rats (except 'infant acquisition). In contrast to all other single brain areas (except IL), the LS was significantly higher activated during 'infant acquisition' compared to 'infant retrieval' (and also compared to 'infant novelty' and 'infant familiarity'). For the adolescent rats, no such difference was revealed. Thus, the 'adolescent acquisition' and 'adolescent retrieval' rats were not significantly different from each other but higher activated than 'adolescent novelty' and 'adolescent

familiarity' (Fig. 18).

Rostral and caudal Hippocampus. In both subregions of the hippocampus, the factor 'condition' had a significant influence on the metabolic activity (Fig. 19).

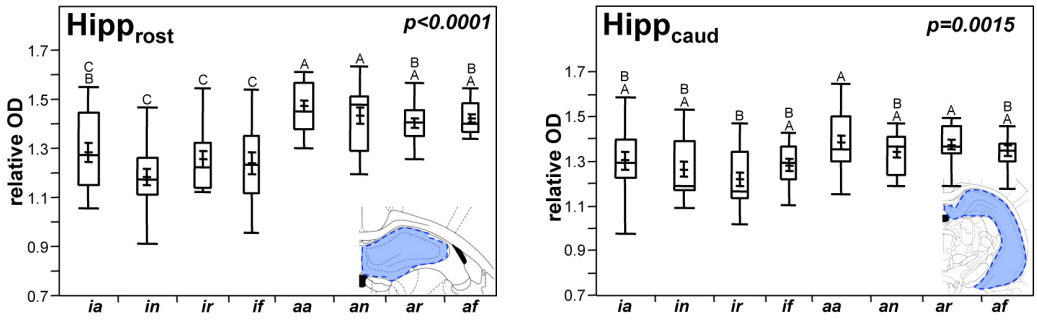


Figure 19: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the $Hipp_{rost}$ and $Hipp_{caud}$ ($p<0.05$).

In the $Hipp_{rost}$, the adolescent rats displayed a higher metabolic activity compared to the infants; however, this difference was only significant for a number of conditions (namely 'adolescent acquisition' and 'adolescent novelty' vs. 'infant novelty', 'infant retrieval' and 'infant familiarity'). Moreover, in the $Hipp_{rost}$, the infant conditions did not differ. Similarly, the adolescent conditions were not different from each other (Fig. 19).

In the $Hipp_{caud}$, the metabolic activities were almost similar between the behavioral conditions. Only 'adolescent acquisition' was significantly higher activated than 'infant retrieval' (Fig. 19).

Infralimbic cortex & Ventro-lateral orbital cortex. Factor 'condition' had a significant influence on the metabolic activity of the IL (Fig. 20; suppl. Tab. 2). In the IL, the adolescent rats displayed a significantly higher metabolic activity than the infant rats (except 'infant acquisition').

Moreover, the IL was significantly higher activated during 'infant acquisition' compared to 'infant retrieval' (also compared to 'infant novelty' but not to 'infant familiarity'). The adolescent conditions were not different from each other (Fig. 20). Thus, the pattern of metabolic activity revealed for the IL was very similar to what was found in the LS.

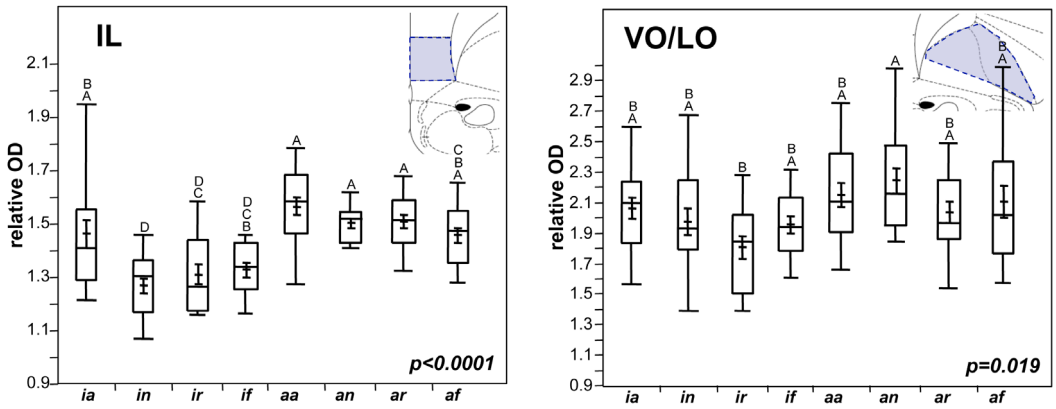


Figure 20: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the IL and VO/LO ($p<0.05$).

In the VO/LO, factor 'condition' had a significant influence on the metabolic activity (Fig. 20). As this was a relatively weak significance, post-hoc comparisons revealed that only 'adolescent novelty' and 'infant retrieval' do significantly differ from each other (Fig. 20).

Amygdaloid subregions. Within the amygdala complex, the factor 'condition' had a significant influence on the metabolic activity of all subregions measured, i.e. BSTI, CeA and MeA and BL (Fig. 21; suppl. Tab. 2).

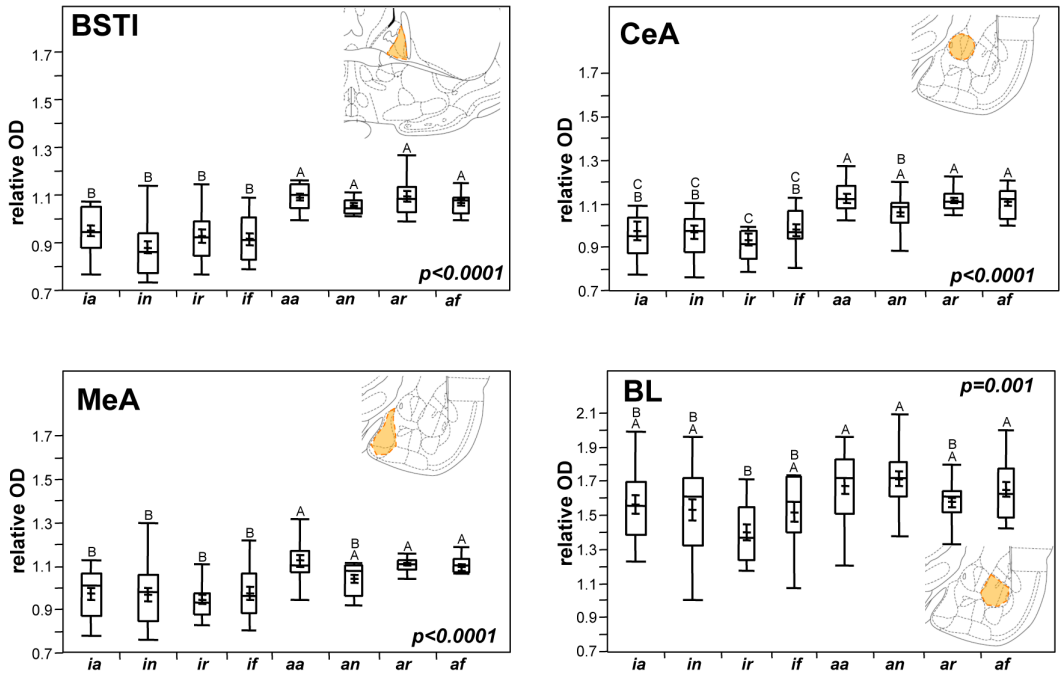


Figure 21: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the BSTI, CeA, MeA and BL ($p<0.05$).

In the BSTI, post-hoc comparisons revealed that the four adolescent conditions did not differ from each other but showed a significant higher metabolic activity than all four infant conditions, which also not differed from each other (Fig. 21).

In the CeA and MeA, post-hoc comparisons revealed a similar pattern, however, with a less sharp difference between adolescent and infant conditions. No differences between acquisition and retrieval conditions of either age were revealed in the CeA and MeA (Fig. 21).

Post-hoc comparisons in the BL revealed a more or less equal metabolic activity between the conditions, only 'infant retrieval' (least metabolic activity) differed significantly from 'adolescent acquisition', 'adolescent novelty' and 'adolescent familiarity' (highest metabolic activity). In both, infants and adolescents, no differences between acquisition and retrieval conditions were revealed in the BL (Fig. 21).

Hypothalamic subregions. Within the hypothalamus, the factor 'condition' had a significant influence on the metabolic activity of all subregions measured, i.e. the LH and VMH (Fig. 22; suppl. Tab. 2).

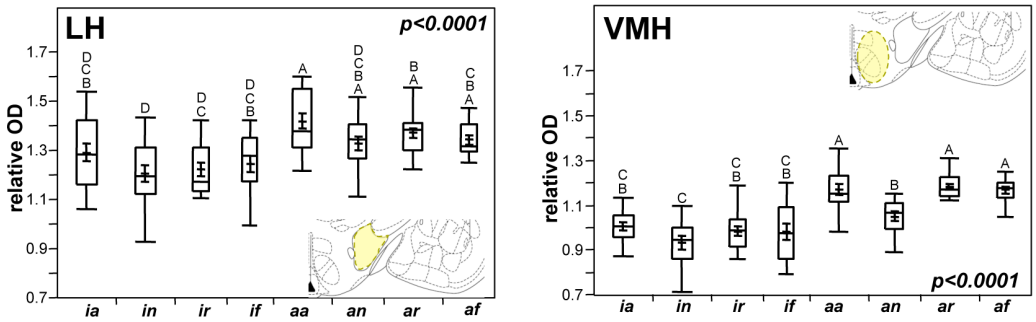


Figure 22: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the LH and VMH (p<0.05).

In the LH, the adolescent conditions generally displayed a higher metabolic activity than the infant conditions. Post-hoc comparisons revealed that there was only a significant difference for the 'adolescent acquisition' compared to all four infant conditions. Adolescent conditions were not different from each other; infant conditions were also not different from each other (Fig. 22).

In the VMH, post-hoc comparisons revealed that 'adolescent acquisition', 'adolescent retrieval' and 'adolescent familiarity' were significantly higher activated than 'adolescent novelty' and all infant conditions. Thus, 'adolescent acquisition', 'adolescent retrieval' and 'adolescent familiarity' were significantly higher activated than 'adolescent novelty' but the metabolic activity in the infant conditions did not differ (Fig. 22).

Brains stem nuclei. Within the brain stem, the factor 'condition' had a significant influence on the metabolic activity of the VTA and PAG but not on the metabolic activity of the DR, MR and IP (Fig. 23; suppl. Tab. 2).

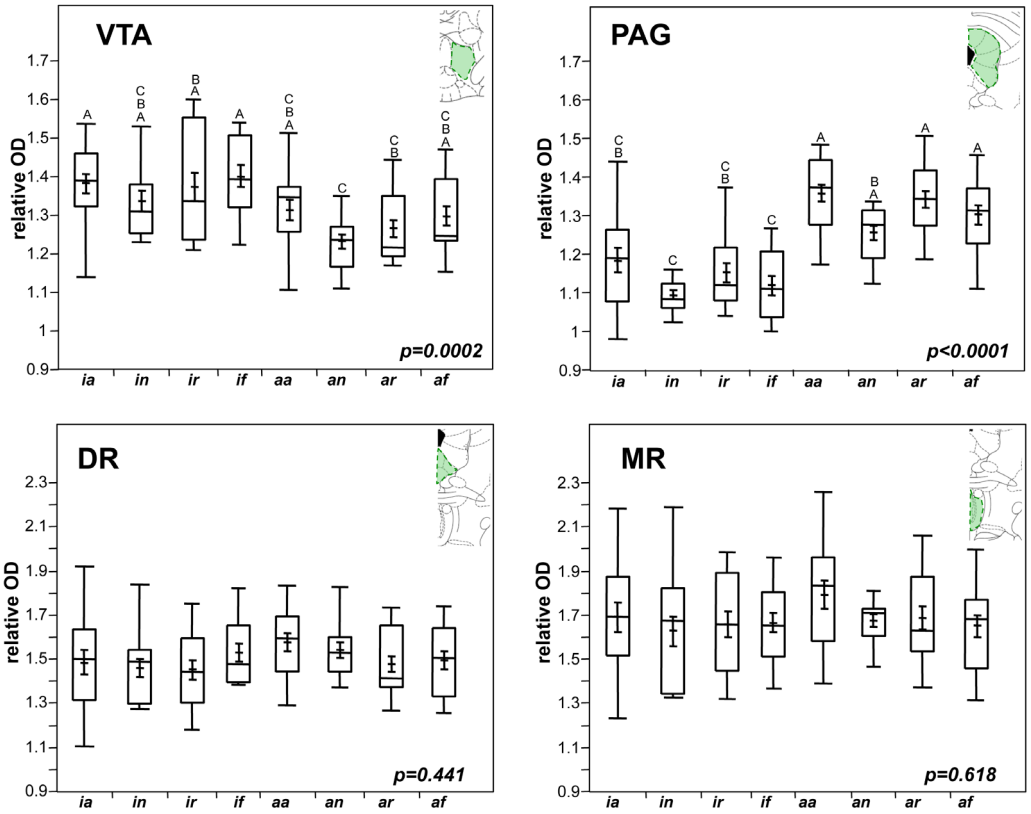


Figure 23: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the VTA, PAG, DR and MR ($p<0.05$).

In the VTA, post-hoc comparisons revealed that – in contrast to all other single brain areas analyzed – the infant conditions displayed either a similar or even higher metabolic activity than the adolescent conditions. Thus, 'infant acquisition' and 'infant familiarity' were significantly higher activated than 'adolescent novelty' and 'adolescent retrieval'. Infant conditions did not differ from each other; also adolescent conditions were similarly activated (Fig. 23).

In the PAG, post-hoc comparisons revealed that the metabolic activity was higher in adolescent rats (except 'adolescent novelty') compared to infant conditions. The metabolic activity in the adolescent conditions did not differ from each other; similarly the metabolic activity in the infant conditions did not differ from each other (Fig. 23).

In the DR and MR, all conditions displayed similar metabolic activities (Fig.

23).

Primary sensory/motor regions. Within the auditory system, the factor 'condition' had a significant influence on the metabolic activity of the Au1 and MG but not on the IC. Moreover, the factor 'condition' had a significant influence on the metabolic activity of PIR, S1HL, S1BF, V1B and M1 (Fig. 24; suppl. Tab. 2).

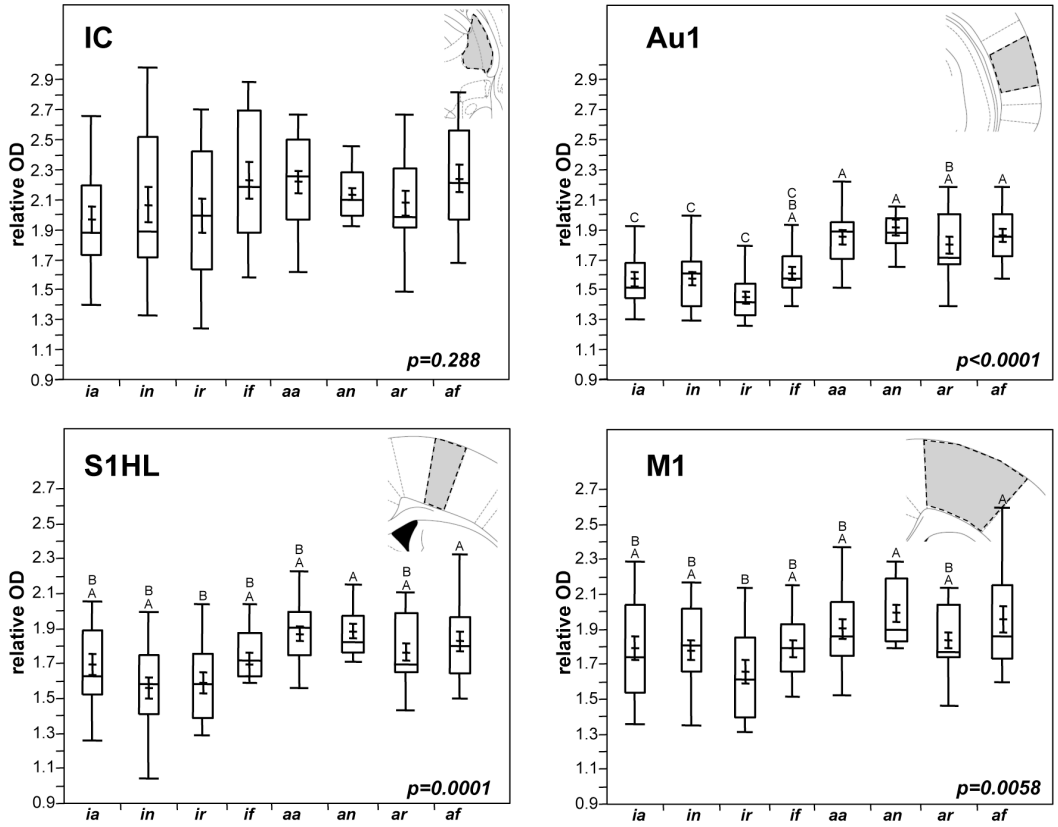


Figure 24: All-in-one comparisons (p-values in bold, italic letters) and pair-wise post-hoc comparisons (significances encoded by capital letters) of metabolic activity in the IC, Au1, S1HL and M1 ($p<0.05$).

In Au1, post-hoc comparisons revealed that the adolescent conditions were significantly higher activated than the infant conditions (except 'infant familiarity'). Adolescent conditions did not differ from each other; likewise, infant conditions did not differ from each other (Fig. 24).

In S1HL, post-hoc comparisons revealed that the metabolic activity was very similar between all conditions. Only 'adolescent novelty' and 'adolescent familiarity' were significantly higher activated than 'infant retrieval'. Identical results were revealed for the M1 (Fig. 24).

3.2.2. Explorative data analysis: Principal component analysis

As outlined in the introduction, the analyses of single regions can only reveal partitioned data sets and results in limited interpretations of these results. Thus, we sought out for differences in the inter-regional correlation patterns reflecting the strengths of metabolic coupling between single brain areas in every single behavioral condition.

First, a principle component analysis (PCA) was applied. This is an exploratory multivariate analysis, which reveals the power of the contribution (defined as 'loading') of the metabolic activity of each single brain area to the variance of the entire data pool. Thus, it enables the investigator to get rid of data, which do not have relevance. In that way, the data amount is reduced and the further analysis can be performed and interpreted more systematical (for details see chapter 2.8).

PCA revealed that 81.32% of the variability of the entire pool of rOD data could be explained by three principal components with 'principal component 1' explaining 65.88% of the variance, 'principal component 2' explaining 10.57 % and 'principal component 3' explaining 4.87 % (Tab. 6). After orthogonal Varimax rotation, the percentage explained by the three principal components was 35.73 % for 'principal component 1', 28.34 % for 'principal component 2' and 17.25 % for 'principal component 3' indicating, that, after rotation, the factors (brain areas) contributing to the principle components became "more similar" in their loading power. Here, we considered areas with loadings ≥ 0.7 relevant for the contribution to the principle components (Bold values in Tab. 6, Veening et al., 2009; Ohl et al., 2003).

'Principal component 1' involved all areas of the limbic cortex (VO/LO, IL, PL, Cg1, Cg1/2 and RSGb) as well as a variety of striatal areas (ACC, ACS, CPdl, CPdm, CPvl), the primary olfactory (PIR), primary motor (M1), primary somato-sensory cortex/sub-region hindlimb (S1HL) and primary visual cortex for binocular vision (V1B). Thus, PC1 was interpreted as the ***cognitive/sensory-motor component***.

'Principal component 2' involved a variety of areas belonging to the hippocampal formation ($Hipp_{rost}$, $Hipp_{caud}$, Sub_{rost}) and amygdala (CeA, MeA) as well as the hypothalamic areas VMH and LH and the brain stem

area PAG. Thus, PC2 was interpreted as the ***emotional-autonomic component***.

'Principal component 3' involved three brain stem areas, i.e. VTA, IP and MR. PC3 was interpreted as the ***modulatory component***.

Region	Area	PC 1: **cognitive/sensory-motor**	PC 2: **emotional-autonomic**	PC 3: **modulatory**
limbic cortex	**VO/LO**	**0.855**		
limbic cortex	**IL**	**0.729**		
limbic cortex	**PL**	**0.900**		
limbic cortex	**Cg1**	**0.892**		
limbic cortex	**Cg1/2**	**0.723**		0.575
limbic cortex	**RSGb**	**0.757**		
hippocampal	**MS/DB**			0.640
hippocampal	**$Hipp_{rost}$**		**0.741**	
hippocampal	**$Hipp_{caud}$**		**0.767**	
hippocampal	**Sub_{rost}**		**0.793**	
hippocampal	**Sub_{caud}**	0.586	0.608	
amygdaloid	**$BSTI_{dp}$**		0.667	
amygdaloid	**CeA**		**0.867**	
amygdaloid	**MeA**		**0.900**	
amygdaloid	**BL**		0.629	0.528
striatal	**ACC**	**0.884**		
striatal	**ACS**	**0.826**		
striatal	**LS**		0.583	
striatal	**CP_{dl}**	**0.776**		
striatal	**CP_{dm}**	**0.701**		
striatal	**CP_{vl}**	**0.702**		
striatal	**CP_{vm}**	0.680		
striatal	**CP_{caud}**		**0.776**	
hypothalamic	**LH**		**0.808**	
hypothalamic	**VMH**		**0.879**	
hypothalamic	**MM**	0.631		0.568
brain stem	**VTA**			**0.879**
brain stem	**IP**			**0.741**
brain stem	**PAG**		**0.711**	
brain stem	**DR**			0.691
brain stem	**MR**	0.516		**0.712**
sensory	**PIR**	**0.701**	0.571	
motor	**M1**	**0.886**		
sensory	**S1HL**	**0.733**		
sensory	**S1BF**	0.571	0.505	
sensory	**Au1**	0.563	0.655	
sensory	**MG**	0.542	0.578	
sensory	**IC**	0.599		0.642
sensory	**V1B**	**0.714**		
%Variance explained		65.775	10.573	4.872
%Variance after factor rotation		**35.727**	**28.342**	**17.250**

Table 6: PCA. The table illustrates the contribution of the single areas (loadings) after Varimax rotation to the three principle components. Loadings ≥ 0.7 (values in bold) and loadings < 0.7 ≥ 0.5 (values in normal characters) are included, whereas loadings < 0.5 are not included. Negative loadings were ≥ -0.073.

3.2.3. Inter-regional correlations of metabolic activity

The metabolic activities of brain areas with PCA-loadings ≥ 0.7 were then correlated. Spearmen's rho coefficients were calculated and visualized as mosaic plots (Fig. 25). Within these plots, red-colored squares indicate significant ($p<0.05$) positive correlations between the rODs of two areas. Thus, if within one individual rat brain, area A displays a high metabolic activity – area B does also display a high metabolic activity. Vice versa, if within another individual rat brain, area A displays a low metabolic activity – area B does also display a low metabolic activity. If this relationship refers to all animals of the same condition, it becomes statistically significant. Light red-colored squares indicate a tendency towards significance ($0.05 \leq p > 0.1$) for positive correlations between the rODs of two brain areas. Blue-colored squares indicate significant ($p<0.05$) negative correlations between the rODs of two areas. Thus, if within one individual rat brain, area A displays a high metabolic activity – area B displays a low metabolic activity. Vice versa, if within another individual rat brain, area A displays a low metabolic activity – area B displays a high metabolic activity. If this relationship refers to all animals of the same condition, it becomes statistically significant. Light blue-colored squares indicate a tendency towards significance ($0.05 \leq p > 0.1$) for negative correlations between the rODs of two areas. Gray-colored squares indicate non-significant correlations between the rODs of two areas ($0.1 \leq p \leq 1$). Thus, there is no stable relationship between the metabolic activities of two areas over the individuals belonging to a behavioral condition.

The following paragraphs (→ ***3.2.3.1.*** to → ***3.2.3.6.***) give a detailed description of the inter-regional correlation patterns. First, the correlations within each principal component (→ ***3.2.3.1.*** to → ***3.2.3.3.***), then the correlations between brain areas of different components (→ ***3.2.3.4.*** to → ***3.2.3.6.***) are described.

The summary – focusing on the most important differences between the inter-regional correlation patterns – is given below. (→ ***3.2.3.7.***).

3.2.3.1. Correlations among brain areas of the cognitive/sensory-motor component (Fig. 25: 1 vs. 1)

Among the brain areas of the cognitive/sensory-motor component, the pattern of the ***'infant acquisition'*** condition was particularly noticeable because –compared to the other seven conditions – the lowest number of positive correlations among the metabolic activities was found here. Thus, the metabolic activity of the IL did not correlate with those of the Cg1/2, RSGb, CPdm, PIR and S1HL. Similarly, the metabolic activity of the Cg1 did not correlate with those of the CPdl, CPdm and PIR. The metabolic activity of the CPvl did not correlate with those of the VO/LO, IL, PL, Cg1 and M1. The metabolic activity of the V1B did not correlate with those of the VO/LO, IL, PL, Cg1, ACC, ACS and M1. In addition, a number of positive correlations showed only a trend towards significance. In contrast, during ***'infant novelty'***, the metabolic activities of all brain areas constituting the cognitive/sensory-motor component were significantly positive correlated with each other. Identically, during ***'infant retrieval'***, the metabolic activities of all brain areas constituting the cognitive/sensory-motor component were significantly positive correlated with each other. During ***'infant familiarity'***, there was also a high degree of inter-regional correlation between the metabolic activities of the brain areas constituting the cognitive/sensory-motor component. However, the metabolic activity of the IL was not correlated with those of the VO/LO, RSGb, CPdl, CPvl, PIR and S1HL. In addition, metabolic activity of the ACS was not correlated with those of the CPdl, CPvl and S1HL. During ***'adolescent acquisition'***, the metabolic activities of almost all brain areas were significantly positive correlated – except those of IL and ACC and those of IL and ACS. During ***'adolescent novelty'***, the metabolic activities of most brain areas were significantly positive correlated with each other. However, the metabolic activity of the IL was not correlated with those of almost all brain areas except PIR. The metabolic activities of ACC and ACS showed only a tendency towards significant positive correlation with the CPdm. During ***'adolescent retrieval'***, the metabolic activities of most brain areas were significantly positive correlated with each other. Only the metabolic activity of the IL was not correlated with those of the Cg1/2, RSGb, ACC, CPvl, S1HL and V1B. Moreover, the metabolic activity of the IL showed only a trend towards a positive correlation with those of ACS and CPdl. During ***'adolescent familiarity'*** all brain areas constituting the cognitive/sensory-motor component were significantly positive correlated with each other.

3.2.3.2. Correlations among brain areas of the emotional-autonomic component (Fig. 25: 2 vs. 2)

Among the brain areas constituting the emotional-autonomic component, most correlations of metabolic activities were significantly positive during ***'infant acquisition'***. A few of them ($Hipp_{rost}$ with CeA, $Hipp_{rost}$ with VHM and Sub_{rost} with VHM showed only a tendency towards significant positive correlations. During ***'infant novelty'***, there were also many significant positive correlations among the metabolic activities of the brain areas constituting the emotional-autonomic component. Only the metabolic activity of the CeA did not correlate with those of the $Hipp_{rost}$ Sub_{rost} and CP_{caud} (by-trend positive

correlation of CeA and $Hipp_{caud}$). Moreover, the metabolic activity of the PAG did not correlate with those of all other brain areas constituting the emotional-autonomic component. Compared to the other behavioral conditions, the metabolic activities of many brain areas constituting the emotional-autonomic components were not correlated with each other during ***'infant retrieval'***. Thus, the metabolic activity of the $Hipp_{rost}$ did not correlate with those of the $Hipp_{caud,}$ Sub_{rost}, CeA and MeA. The metabolic activity of the CeA did not correlate at all with that of the CP_{caud} and did only show a tendency towards significant positive correlation with that of the Sub_{rost}. The metabolic activity of the MeA did not correlate with those of the $Hipp_{rost}$, $Hipp_{caud}$, Sub_{rost} and PAG. The metabolic activity of the VMH did not correlate with those of the $Hipp_{caud,}$ Sub_{rost} and CP_{caud}. Moreover, the metabolic activity of the PAG did not correlate with those of the $Hipp_{caud,}$ Sub_{rost}, CeA, MeA CP_{caud}. During ***'infant familiarity'***, only the metabolic activity of the $Hipp_{caud}$ did not correlate with that of the Sub_{rost}. There were also some brain areas that displayed only a tendency towards significant positive correlation of their metabolic activities (i.e. Sub_{rost} vs. $Hipp_{rost}$, MeA and VMH; PAG vs. CeA and MeA). During ***'adolescent acquisition'***, the metabolic activities of almost all brain areas were significantly positive correlated with each other. Only the metabolic activity of the PAG was not at all or not significantly correlated with those of the VMH and MeA, respectively. During ***'adolescent novelty'***, the metabolic activity of the Sub_{rost} was not correlated with those of CP_{caud} and PAG. The metabolic activity of the VMH was not correlated with those of the $Hipp_{rost}$ and CP_{caud} and only showed a trend towards a significant positive correlation with those of the $Hipp_{caud}$, CeA and LH. Moreover, the metabolic activity of the PAG was not significantly correlated with those of the Sub_{rost}, MeA and VMH. The metabolic activities of all other brain areas were significantly positive correlated during 'adolescent novelty'. Also many significant positive correlations were found during ***'adolescent retrieval'***. The metabolic activity of the CeA was not correlated with those of the $Hipp_{rost}$ and CP_{caud} and it showed a trend towards a significant positive correlation with those of the Sub_{rost}, LH and PAG. There were also some brain areas that displayed only a tendency towards significant correlation of their metabolic activities (i.e. Sub_{rost} vs. VMH; PAG vs. MeA). During ***'adolescent familiarity'***, there were a number of metabolic activities of brain areas that were not correlated. Thus, the metabolic activity of the CeA was not correlated with those of the $Hipp_{rost}$, $Hipp_{caud,}$ $Sub_{rost,}$ CP_{caud} and PAG. The metabolic activity of the MeA was not correlated with those of the $Hipp_{caud,}$ Sub_{rost}, CP_{caud}, LH and VMH and showed a trend towards a significant negative correlation with that of the PAG. The metabolic activity of the VMH was not correlated with those of the $Hipp_{rost}$, $Hipp_{caud,}$ Sub_{rost}, MeA, CP_{caud}, LH and PAG.

3.2.3.3. Correlations among brain areas of the modulatory component (Fig. 25: 3 vs. 3)

During all eight behavioral conditions, the metabolic activities of the three brain areas constituting the modulatory component were significantly positive correlated with each other.

3.2.3.4. Correlations of brain areas of the cognitive/sensory-motor component vs. brain areas of the emotional-autonomic component (Fig. 25: 1 vs. 2)

Among the brain areas constituting the cognitive/sensory-motor and emotional-autonomic components, there were a high number of metabolic activities of brain regions not correlated during ***'infant acquistion'***. Thus, the metabolic activities of all brain areas of the emotional-autonomic component were not correlated with those of the VO/LO, IL, PL, Cg1, ACC, ACS and M1. In addition, the metabolic activity of the CeA was not correlated with those of the CPdl, CPvl and PIR and it only showed a trend towards significance with those of Cg1/2, RSGb, CPdm and S1HL. Similarly, the metabolic activity of the MeA was not correlated with those of the CPdl, CPdm, CPvl and PIR it only showed a trend towards significance with those of the Cg1/2, RSGb and S1HL. The metabolic activity of the VMH was not correlated with those of the Cg1/2, RSGb, CPdl, CPdm, CPvl, PIR and S1HL. During ***'infant novelty'***, there were many significant positive correlations among the brain areas constituting the cognitive/sensory-motor and emotional-autonomic components except for the CeA, MeA and PAG. In detail, the metabolic activity of the $Hipp_{caud}$ did not correlate with that of the PL and only showed a trend towards significance with that of the CPdm. The metabolic activity of the Sub_{rost} did not correlate with those of the IL and PL and only showed a trend towards significance with that of the ACS. The metabolic activity of the CeA did not correlate with those of most brain regions constituting the cognitive/sensory-motor component (except VO/LO and M1 – here a trend towards significant positive correlations was observed). The metabolic activity of the MeA did not correlate with those of the IL, PL, ACC, ACS, CPdm and CPvl and only showed a trend towards significance with those of the Cg1/2 and CPdl. The metabolic activity of the CP_{caud} did not correlate with those of the IL and PL and only showed a trend towards significance with that of the CPdm. The metabolic activity of the PAG did not correlate with those of most brain regions constituting the cognitive/sensory-motor component (except V1B – here a trend towards significant positive correlation was observed). During '***infant retrieval'***, the metabolic activities of $Hipp_{caud,}$ Sub_{rost}, CeA and MeA did entirely not correlate with those of all brain areas constituting the cognitive/sensory-motor component. Moreover, the metabolic activity of the CP_{caud} did not correlate with those of the IL, CPvl and S1HL and only showed a trend towards a significant positive correlation with those of the VO/LO, Cg1, Cg1/2, ACC, ACS, CPdl, CPdm, PIR, M1 and V1B. The metabolic activity of the LH showed only a trend towards a significant positive correlation with those of the Cg1/2, RSGb and S1HL. The metabolic activity of the VMH did not correlate with those of the Cg1/2, RSGb, CPdm, CPvl and M1 and only showed a trend towards a significant positive correlation with those of the PL, ACC, CPdl and S1HL. The metabolic activity of the PAG did not correlate with that of the CPdm and showed only a trend towards a significant positive correlation with those of the IL and CPvl. Thus, the number of significantly positve correlated brain regions was very low between these two components during 'infant retrieval'. Also during ***'infant familiarity'***, the number of significantly positive correlated brain regions was very low between these two components. However, the pattern of significant vs. non-significant inter-regional correlations was much more scattered compared to 'infant retrieval'. In

detail, the metabolic activity of the $Hipp_{caud}$ did not correlate with those of the VO/LO, RSGb, CPdl, CPvl, M1 and S1HL. The metabolic activity of the Sub_{rost} did not correlate with those of the VO/LO, IL, PL, Cg1, RSGb, CPdl, CPdm, CPvl, M1 and S1HL and only showed a trend towards a significant positive correlation with those of the Cg1/2, ACC, ACS and PIR. The metabolic activity of the CeA did not correlate with those of the VO/LO, RSGb, ACC, ACS, CPdl, CPvl, M1 and S1HL and only showed a trend towards a significant positive correlation with those of the IL and CPdm. The metabolic activity of the MeA did not correlate with those of the VO/LO, RSGb, ACC, ACS, CPdl, CPvl, M1 and S1HL and only showed a trend towards a significant positive correlation with those of the IL and CPdm. The metabolic activity of the LH did not correlate with that of the CPdl and only showed a trend towards a significant positive correlation with those of the VO/LO and S1HL. The metabolic activity of the VMH did not correlate with those of the VO/LO, Cg1, RSGb, ACC, CPdl, CPvl, M1 and S1HL. Similarly, The metabolic activity of the PAG did not correlate with those of the VO/LO, Cg1, RSGb, ACC, CPdl, M1 and S1HL and only showed a trend towards a significant positive correlation with those of the PL, CPdm and CPvl. During ***'adolescent acquisition'***, CeA, MeA and VMH diplayed very similar inter-regional correlation patterns. Moreover, the metabolic activity of the IL was significantly positive correlated with each of the brain areas constituting the emotional-autonomic component. In detail, the metabolic activity of the $Hipp_{rost}$ did not correlate with those of the ACC and ACS and showed a tendency towards significant positive correlation with that of the V1B. The metabolic activity of the $Hipp_{caud}$ did not correlate with those of the VO/LO, Cg1/2, RSGb, ACC, ACS, M1 and V1B but showed a tendency towards significant positive correlation with that of the PL, Cg1 and S1HL. The metabolic activity of the Sub_{rost} did not correlate with those of the Cg1, ACC, ACS M1 and V1B and only showed a trend towards a significant positive correlation with those of the VO/LO, Cg1/2 and RSGb. The metabolic activity of the CeA did not correlate with those of the VO/LO, PL, Cg1, Cg1/2, RSGb, ACC, CPdm, CPdl, CPvl, PIR, M1 and S1HL and only showed a trend towards a significant positive correlation with that of the V1B as well as a trend towards significant negative correlation with that of the ACS. The metabolic activity of the MeA did not correlate with those of the VO/LO, PL, Cg1, Cg1/2, RSGb, ACC, ACS, CPdm, CPdl, CPvl, PIR, M1, S1HL and V1B. The metabolic activity of the CP_{caud} did not correlate with those of the ACC, ACS and V1B and only showed a trend towards a significant positive correlation with those of the Cg1, Cg1/2, RSGb and M1. Similarly, the metabolic activity of the LH did not correlate with those of the Cg1/2, RSGb, ACC, ACS and V1B and only showed a trend towards a significant positive correlation with those of the VO/LO, Cg1 and M1. The metabolic activity of the VMH did not correlate with those of the VO/LO, PL, Cg1, Cg1/2, RSGb, ACC, CPdm, CPdl, CPvl, PIR, M1, S1HL and V1B but showed a significant negative correlation with that of the ACS. Finally, the metabolic activity of the PAG did not correlate with that of the ACS and only showed a trend towards a significant positive correlation with that of the ACC. ***'Adolescent novelty'*** displayed many inter-regional correlations that were either non-significant or even significantly negative. In detail, the metabolic activity of the $Hipp_{rost}$ did not correlate with those of the IL, Cg1/2, RSGb, ACC, ACS, M1 and S1HL and showed a tendency towards significant

positive correlation with those of the Cg1, PL, CPdl and V1B. The metabolic activity of the $Hipp_{caud}$ did not correlate with those of the IL, Cg1, Cg1/2, RSGb, ACC, ACS, CPdl, M1 and V1B but showed a tendency towards significant positive correlation with those of the VO/LO, PL, and CPvl. The metabolic activities of both - the Sub_{rost} and MeA – did not correlate with those of the VO/LO, IL, PL, Cg1, Cg1/2, RSGb, ACC, ACS, CPdl, CPvl, PIR, M1, S1HL and V1B and only showed a trend towards a significant positive correlation with that of the CPdm. The metabolic activity of the CeA did not correlate with those of the, IL, PL, Cg1/2, RSGb, ACC, CPdl, M1, S1HL and V1B and showed a trend towards a significant positive correlation with that of the VO/LO, Cg1 and CPvl. The metabolic activity of the CP_{caud} did not correlate with those of the IL, ACC, ACS and S1HL and only showed a trend towards a significant positive correlation with those of the PL, Cg1/2, M1 and V1B. Similarly, the metabolic activity of the LH did not correlate with those of the IL, Cg1/2, ACC, ACS S1HL and V1B and only showed a trend towards a significant positive correlation with those of the PL, Cg1, RSGb, CPvl and M1. The metabolic activity of the VMH did not correlate with those of the VO/LO, IL, PL, Cg1, RSGb, CPdm, CPvl, PIR and V1B but showed significant negative correlations with those of the Cg1/2, ACC, ACS and S1HL. Moreover, the metabolic activity of the VMH showed a tendency towards a significant negative correlation with those of the CPdl and M1. Finally, the metabolic activity of the PAG significantly positive correlated with those of almost all brain areas constituting the cognitive/sensory-motor component except S1HL (trend towards significant positive correlation. During '***adolescent retrieval'*** a high number of significant positive correlations between the brain regions of the cognitive/sensory-motor and the emotional-autonomic components were found – except for the IL (no correlation at all!), CeA and VMH. Thus, beside the IL, metabolic activity of the Sub_{rost} did not correlate with that of the V1B. The metabolic activity of the CeA did also not correlate with that of any of the brain areas – except CPvl (tendency towards significant positive correlation). The metabolic activity of the MeA did not correlate with those of the VO/LO, IL, PL and M1, S1HL and V1B and only showed a trend towards a significant positive correlation with those of the ACS, PIR, S1HL and V1B. The metabolic activity of the VMH did not correlate with those of the VO/LO, IL, PL, CPdl and M1, S1HL and V1B and only showed a trend towards a significant positive correlation with those of the ACS, PIR, S1HL and V1B. ***Adolescent familiarity'*** was the only condition where a number of negative correlations were consistently found between the metabolic activities of both regions – CeA and MeA – and those of the brain areas of the cognitive/sensory-motor component. In detail, $Hipp_{rost}$ showed significant positive correlations with all other brain areas except V1B. The metabolic activity of the $Hipp_{caud}$ did not correlate with those of the VO/LO, ACS, M1 and V1B but showed a tendency towards significant positive correlation with that of the PL, Cg1/2, RSGb, CPdl, PIR and S1HL. Similarly, metabolic activity of the Sub_{rost} did not correlate with those of the ACS and V1B but showed a tendency towards significant positive correlation with those of the VO/LO, PL, Cg1, Cg1/2, RSGb, CPdl, PIR, M1 and S1HL. The metabolic activities of both – the CeA and MeA – did not correlate positively with any of the brain areas constituting the cognitive/sensory-motor component. Rather, the metabolic activity of the CeA correlated significantly negative with

those of the VO/LO, Cg1, RSGb, ACS, CPdl, M1, S1HL, V1B and it showed a trend towards a significant negative correlation for PL, Cg1/2, CPdm and PIR. No correlations of the metabolic activity of the CeA were revealed for those of the IL, ACC and CPvl. The metabolic activity of the MeA correlated significantly negative with those of the VO/LO, Cg1, Cg1/2, RSGb, ACS, CPdl, PIR, M1 and S1HL and it showed a trend towards a significant negative correlation with those of the PL and V1B. No correlations of the metabolic activity of the CeA were revealed for the IL, ACC, CPdm and CPvl. The CP_{caud} showed significant positive correlations with all other brain areas except CPdl and V1B (trends towards significant positive correlation). The metabolic activity of the LH did not correlate with those of the VO/LO, Cg1, Cg1/2, CPdl, CPvl, PIR, M1 and S1HL and V1B but showed a tendency towards significant positive correlation with those of the PL and RSGb. VMH did not show any correlation (except with the V1B – tendency towards significant negative correlation). The metabolic activity of the PAG correlated significantly positive with those of all brain regions constituting the cognitive/sensory-motor component.

3.2.3.5. Correlations of brain areas of the cognitive/sensory-motor component vs. brain areas of the modulatory component (Fig. 25: 1 vs. 3)

During 'infant acquisition', there were a number of metabolic activities of brain areas constituting the cognitive/sensory-motor and modulatory components, which were not correlated. Thus, the metabolic activities of both regions – the VTA and MR – were not correlated with those of the VO/LO, IL, PL, Cg1, ACC and ACS as well as M1. Moreover, the metabolic activity of the IP positively correlated with the brain areas constituting the cognitive/sensory-motor component, but it only showed a trend towards significance with those of IL, Cg1 and CPvl. During ***'infant novelty'***, the metabolic activities of all brain areas constituting the cognitive/sensory-motor component were significantly positive correlated with those of all brain areas constituting the modulatory component. Also during ***'infant retrieval'***, the metabolic activities of all brain areas constituting the cognitive/sensory-motor and modulatory components were significantly positive correlated with each other. During ***'infant familiarity'***, the metabolic activity of the VTA was not correlated with those of the VO/LO, Cg1, RSGb, CPdl, and S1HL and only showed a trend towards significant positive correlation with those of the CPvl and M1. Moreover, metabolic activity of the IP was not correlated with those of the VO/LO, CPdl, and S1HL. During ***'adolescent acquisition'***, the metabolic activities of almost all brain areas constituting the cognitive/sensory-motor and modulatory components were significantly positive correlated with each other – except ACS vs. IP (trend towards significant positive correlation). During ***'adolescent novelty'***, there were a high number of significant positive correlations. The metabolic activity of the IL did not correlate with that of the IP and showed only a tendency towards a significant positive correlation with the VTA. The metabolic activity of the ACC did not correlate with the MR and showed only a tendency towards a significant positive correlation with the VTA. The metabolic activity of the ACS did not correlate with the VTA, IP and MR. Also during ***'adolescent retrieval'***, the metabolic activities of almost all brain areas constituting the

cognitive/sensory-motor and modulatory components were significantly positive correlated with each other – except IL vs. VTA. During ***'adolescent familiarity'***, the metabolic activities of all brain areas constituting the cognitive/sensory-motor and modulatory components were significantly positive correlated with each other.

3.2.3.6. Correlations of brain areas of the emotional-autonomic component vs. brain areas of the modulatory component (Fig. 25: 2 vs. 3)

During ***'infant acquisition'***, a high number of significant positive correlations were found among the brain areas constituting the emotional-autonomic and modulatory components. The metabolic activity of the IP showed only a tendency towards a significant positive correlation with the MeA and VMH. The metabolic activity of the MR did not correlate with those of the MeA and VMH and showed only a tendency towards a significant positive correlation with that of the CeA. During ***'infant novelty'***, also many significant positive correlations were found among the brain areas constituting the emotional-autonomic and modulatory components. The metabolic activity of the CeA did not correlate with that of the VTA and showed only a tendency towards a significant positive correlation with the MR. The metabolic activity of the PAG did not correlate with that of the VTA and IP and showed only a tendency towards a significant positive correlation with the MR. During ***'infant retrieval'***, there were a number of metabolic activities of brain areas constituting the emotional-autonomic and modulatory components, which were not correlated. Thus, the metabolic activities of the Sub_{rost}, CeA and MeA did identically not correlate with those of the VTA, IP and MR. Moreover, the metabolic activity of the $Hipp_{caud}$ did not correlate with that of the MR and showed only a tendency towards a significant positive correlation with the VTA and IP. The metabolic activity of the CP_{caud} did not correlate with that of the MR. The metabolic activity of the VMH did not correlate with that of the IP and the metabolic activity of the PAG did not correlate with that of the VTA. During ***'infant familiarity'***, a high number of significant positive correlations were found among the metabolic activities of the brain areas constituting the emotional-autonomic and modulatory components. The metabolic activity of the Sub_{rost} did not correlate with that of the IP and showed only a tendency towards a significant positive correlation with the VTA. The metabolic activity of the MeA showed only a tendency towards a significant positive correlation with that of the MR. During ***'adolescent acquisition'***, the metabolic activities of the CeA, MeA and VMH did not correlate with those of the VTA, IP and MR (in case of CeA vs. VTA it was a trend towards significant positive correlation). The metabolic activities of all other brain areas constituting the emotional-autonomic and modulatory components were significantly positive correlated. A similar pattern arose during ***'adolescent novelty'***. Thus, there were many significant positive correlations among the brain areas constituting the emotional-autonomic and modulatory components. However, the metabolic activity of the Sub_{rost} did not correlate with that of the MR, the metabolic activity of the MeA did not correlate with those of the VTA and IP and the metabolic activity of the VMH did not correlate with that of the VTA, IP and MR. During ***'adolescent retrieval'***, also a high number of significant positive correlations among the brain areas constituting the emotional-autonomic and

modulatory components were found. Again, the metabolic activity of the CeA did not correlate with those of the VTA, IP and MR. The metabolic activity of the MeA did not correlate with that of IP and showed a trend towards significant positive correlation with those of the VTA and MR. The metabolic activity of the VMH showed a trend towards significant positive correlation with that of the MR. During ***'adolescent familiarity'***, the most diffuse correlation pattern was found among the metabolic activities of the brain areas constituting the emotional-autonomic and modulatory components. Thus, the metabolic activity of the $Hipp_{caud}$ did not correlate with that of IP and showed a trend towards significant positive correlation with that of the MR. The metabolic activity of the Sub_{rost} showed a trend towards significant positive correlation with those of the VTA and IP. The metabolic activity of the CeA did significantly negative correlate with that of IP and showed a trend towards significant negative correlation with those of the VTA and MR. The metabolic activity of the MeA did 1) significantly negative correlate with that of VTA, 2) showed a trend towards significant negative correlation with those of the IP and 3) did not at all correlate with that of the MR. The metabolic activity of the LH did not correlate with that of VTA and showed a trend towards significant positive correlation with those of the IP and MR. Finally, the metabolic activity of the VMH did not correlate with that of VTA, IP and MR.

3.2.3.7. Summary of results revealed by inter-regional correlations of metabolic activities

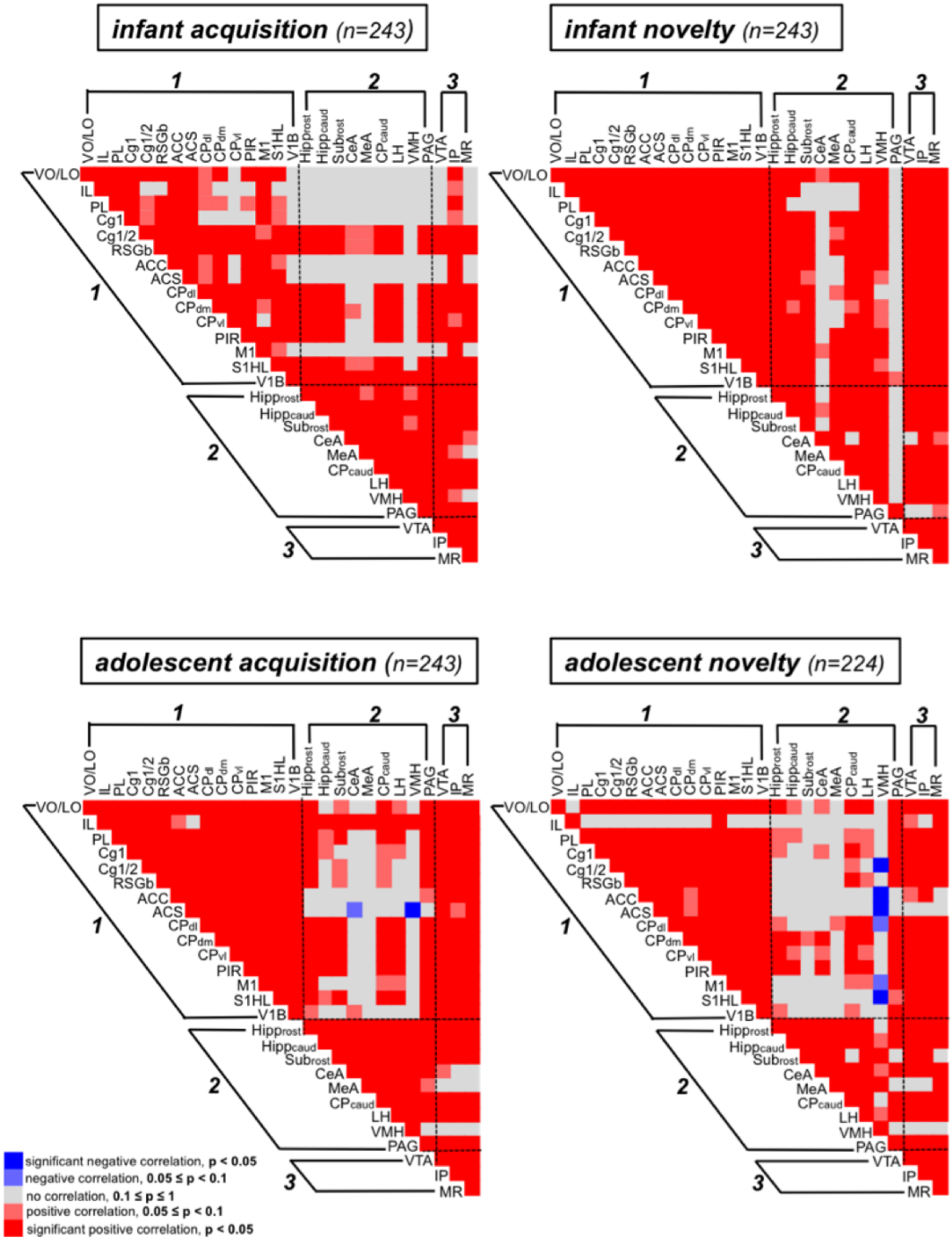
The general pattern of inter-regional correlations was relatively similar between the groups. Basically, significant positive correlations (red squares) between metabolic activities represented the most frequent type of statistical relationship among the brain regions in every behavioral condition. However, there were also brain areas that displayed significant negative (blue squares), by-trend (light blue/ light red) or no correlations (gray squares) of their metabolic activities. The number and distribution of these negative, by-trend and non-significant correlations varied between the behavioral conditions.

If the three principal components were considered separately, there was either complete significant positive correlation or a high number of significant positive correlations among the metabolic activities of the brain regions constituting each of these three components (→ ***3.2.3.1.*** to → ***3.2.3.3.***). However, among the brain regions constituting the cognitive/sensory-motor component, the lowest number of positive correlations of metabolic activities was found in the 'infant acquisition' condition. All other behavioral conditions consistently displayed a

complete or high degree of significant positive correlations among the metabolic activities of the brain regions constituting the cognitive/sensory-motor component (except IL during ‘infant familiarity’, ‘adolescent novelty’ and adolescent retrieval. Moreover, during ‘infant retrieval’ and ‘adolescent familiarity’, the brain regions constituting the emotional-motor component also frequently displayed metabolic activities that were not correlated with each other. The metabolic activities of the three brain areas constituting the modulatory component were significantly positive correlated with each other during all eight behavioral conditions.

Strikingly, the metabolic activities of a varying number of brain regions constituting the emotional-autonomic component were not, or even negatively (‘adolescent novelty and ‘adolescent familiarity’), correlated with the metabolic activities of the brain regions constituting the cognitive/sensory-motor component (→ ***3.2.3.4.***). Similarly, the metabolic activities of a varying number of brain regions constituting the emotional-autonomic component were not, or even negatively (‘adolescent novelty and ‘adolescent familiarity’), correlated with the metabolic activities of the brain regions constituting the modulatory component (→ ***3.2.3.6.***).

During ‘adolescent retrieval’, i.e. in the rats that displayed TWA behavior after five days of training, there were only a few non-correlated metabolic activities. Namely, the metabolic activities of the IL and the CeA (partly MeA and VMH) did not correlate with those of most other brain regions. In comparison, during ‘infant retrieval’, i.e. in the rats that did not display TWA behavior despite five consecutive days of training, the metabolic activities of $Hipp_{caud}$, Sub_{rost}, CeA and MeA almost consistently not correlated with those of all other brain regions.


infant acquisition (n=243)
infant novelty (n=243)
adolescent acquisition (n=243)
adolescent novelty (n=224)
1
2
3
VO/LO
IL
PL
Cg1
Cg1/2
RSGb
ACC
ACS
CPdl
CPdm
CPvl
PIR
M1
S1HL
V1B
Hipprost
Hippcaud
Subrost
CeA
MeA
CPcaud
LH
VMH
PAG
VTA
IP
MR
significant negative correlation, p < 0.05
negative correlation, 0.05 ≤ p < 0.1
no correlation, 0.1 ≤ p ≤ 1
positive correlation, 0.05 ≤ p < 0.1
significant positive correlation, p < 0.05

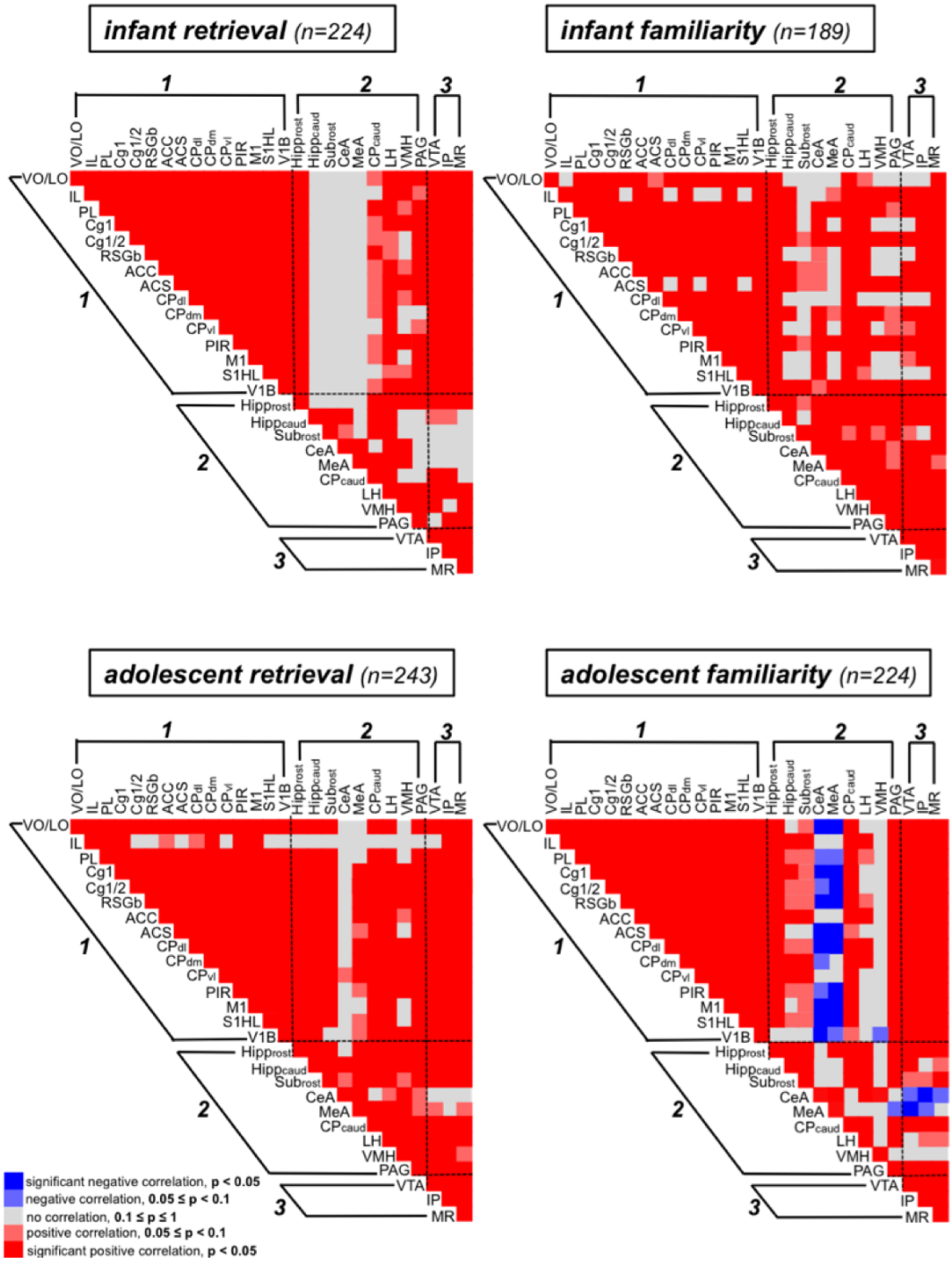


Figure 25: Inter-regional correlations of metabolic activity (i.e. "Correlated functional activity"). Altogether, 1609 rODs (calculated from 38860 by-hand measurements) were included in the analysis. Number of rODs per condition is given in brackets, respectively. 1=cognitive/sensory-motor component, 2=emotional-autonomic component, 3=modulatory component.

3.3. Correlations of behavioral parameters and metabolic activity

Spearmen's rho coefficients were calculated to search for statistical dependencies between the metabolic activity and the behavioral parameters monitored during TWA training. The coefficients were also visualized as mosaic plots (Fig. 26).

Red-colored squares indicate significant ($p<0.05$) positive correlations between the rODs a brain area and a behavioral parameter. Thus, if within one individual rat brain, area A displays a high metabolic activity – the number of avoidance reactions (or another parameter) is also high. Vice versa, if within another individual rat brain, area A displays a low metabolic activity – the number of avoidance reactions would be also low. If this relationship refers to all animals of the same condition, it becomes statistically significant.

Light red-colored squares indicate a tendency towards significance ($0.05 \leq p > 0.1$) for positive correlations between the rODs of a brain area and the behavioral performance of the individuals within one condition.

Blue-colored squares indicate significant ($p<0.05$) negative correlations between the rODs of two areas. Thus, if within one individual rat brain, area A displays a high metabolic activity –the number of avoidance reactions (or another parameter) is low. Vice versa, if within another individual rat brain, area A displays a low metabolic activity – the number of avoidance reactions is high. If this relationship refers to all animals of the same condition, it becomes statistically significant.

Light blue-colored squares indicate a tendency towards significance ($0.05 \leq p > 0.1$) for negative correlations between the rODs of a brain area and the behavioral performance of the individuals within one condition.

Gray-colored squares indicate non-significant correlations between the rODs of a brain area and the behavioral performance ($0.1 \leq p \leq 1$).

The following paragraphs (→ ***3.3.1.*** to → ***3.3.3.***) give a very detailed description of the correlation patterns.

The summary – focusing on the most important differences between the four training conditions – is given below. (→ ***3.3.4.***).

3.3.1. Correlations between behavioral parameters and the metabolic activities of cognitive/ sensory-motor areas

During ***'infant acquisition'***, there were only some scattered correlations found. Thus, the number of avoidances correlated significantly negative with the metabolic activities of the IL, ACC and ACS and showed a trend towards significant negative correlation with those of the VO/LO and M1. The number of escapes showed a trend towards significant negative correlation with the metabolic activity of the Cg1 and the number of failures was significantly positive correlated with the metabolic activities of the VO/LO, IL, PL, Cg1, ACC and ACS. No correlations were revealed for the UCS exposure time. During ***'infant retrieval'***, the numbers of avoidances and escapes were not correlated with the metabolic activity of any brain area of the cognitive/sensory-motor component. In contrast, the number of failures and the UCS exposure times were significantly negative correlated with almost all metabolic activities of all brain areas constituting the cognitive/sensory-motor component (except PL, Cg1/2 and RSGb for UCS exposure on P21). During ***'adolescent acquisition',*** no correlations were revealed for the number of avoidances. The number of escapes did significantly negative correlate with the metabolic activity of the IL. The number of failures did significantly positive correlate with the metabolic activity of the IL and showed a trend towards significant positive correlation with that of the ACS. Also the UCS exposure time did only significantly positive correlate with the metabolic activity of the IL. During ***'adolescent retrieval'***, the number of avoidances significantly negative correlated with the metabolic activities of VO/LO, PL, RSGb, ACS, CPvl, M1, S1HL and V1B and showed a trend towards a significant negative correlation with the metabolic activities of the Cg1 and CPdl. Vice versa, the number of escapes significantly positive correlated with the VO/LO, PL, RSGb, ACS, CPvl, M1, S1HL and V1B and showed a trend towards a significant positive correlation with the metabolic activities of the Cg1 and CPdl. The UCS exposure time on P42 showed a significant positive correlation with the metabolic activity of the M1 and a trend towards significant positive correlations with the metabolic activities of the IL, Cg1 and S1HL. The cumulative UCS exposure time on P42 showed a significant positive correlation with the metabolic activity of the IL.

3.3.2. Correlations between behavioral parameters and the metabolic activities of emotional-autonomic areas

During ***'infant acquisition'***, there were no correlations found for the numbers of avoidances, escapes and failures. The UCS exposure time correlated significantly negative with the metabolic activities of the $Hipp_{rost}$, CP_{caud} and LH.

During ***'infant retrieval'***, the number of avoidances significantly positive correlated with the metabolic activity of the Sub_{rost} and showed a trend towards a significant positive correlation with that of the $Hipp_{caud}$. The number of escapes significantly negative correlated with the metabolic activity of the Sub_{rost} and showed a trend towards a significant negative correlation with that of the $Hipp_{caud}$. The number of failures significantly negative correlated with the metabolic activity of the LH and showed a trend towards a significant negative correlation with that of the VMH. The UCS exposure time

on P21 showed a trend towards a significant negative correlation with the metabolic activities of the LH and VMH. The cumulative UCS exposure time significantly negative correlated with the metabolic activity of the LH and VMH and showed a trend towards a significant negative correlation with that of the MeA and CP_{caud}. During ***'adolescent acquisition'***, the number of avoidances significantly positive correlated with the metabolic activity of the VMH and showed a trend towards a significant positive correlation with that of the MeA. The number of escapes significantly negative correlated with the metabolic activity of the $Hipp_{caud}$, CeA, MeA, LH and VMH and showed a trend towards a significant negative correlation with that of the Sub_{rost} and CP_{caud}. The number of failures significantly positive correlated with the metabolic activity of all brain regions of the emotional-autonomic component. The UCS exposure time on P42 significantly positive correlated with the metabolic activity of the $Hipp_{rost}$, Sub_{rost}, CeA, LH and PAG and showed a trend towards a significant positive correlation with the metabolic activities of the $Hipp_{caud}$, MeA and CP_{caud}. During ***'adolescent retrieval'***, the number of avoidances significantly negative correlated with the metabolic activities of the $Hipp_{caud}$ and Sub_{rost}. Vice versa, the number of escapes significantly positive correlated with the metabolic activities of the $Hipp_{caud}$ and Sub_{rost}. The UCS exposure times did not show any correlations with the metabolic activity.

3.3.3. Correlations between behavioral parameters and the metabolic activities of modulatory areas

During ***'infant acquisition'***, there were no correlations found for the numbers of avoidances, escapes and failures. The UCS exposure time correlated significantly negative with the metabolic activity of the MR and showed a tendency towards significant negative correlation with that of the IP. During ***'infant retrieval'***, the numbers of avoidances and escapes were not significantly correlated with the metabolic activity of any brain area of the cognitive/sensory-motor component (only no. of avoidances and IP showed a tendency towards significant positive correlation. In contrast, the number of failures and the UCS exposure times were significantly negative correlated with the metabolic activities of all brain areas constituting the modulatory component. During ***'adolescent acquisition'***, there were no correlations found for the numbers of avoidances and escapes. The number of failures showed a tendency towards significant positive correlation with the metabolic activity of the MR. The UCS exposure time correlated significantly positive with the metabolic activity of the MR. During ***'adolescent retrieval'***, the number of avoidances significantly negative correlated with the metabolic activities of the IP and MR and showed a trend towards a significant positive correlation with that of the VTA. Vice versa, the number of escapes significantly positive correlated with the metabolic activities of the IP and MR and showed a trend towards a significant negative correlation with that of the VTA. The UCS exposure times did not show correlations with the metabolic activity (except for the UCS exposure on P42 and the metabolic activity of the IP).

3.3.4. Summary of results revealed by correlations of behavioral parameters and metabolic activities

During ‘infant acquisition’, only some scattered correlations were found. In contrast, during ‘infant retrieval’ almost no correlations were found for the numbers of avoidances and escapes. However, a consistent negative correlation of the number of failures and UCS exposure times and the metabolic activities of the brain areas constituting the cognitive/sensory-motor and the modulatory components was revealed. Within the emotional-autonomic component, the metabolic activities of the LH and VMH showed a similar negative correlation with the number of failures and UCS exposure times.

During ‘adolescent acquisition’ the striking result was that almost all metabolic activities of the brain areas constituting the emotional-autonomic component as well as that of the IL were negatively correlated with the number of escapes and positively correlated with the number of failures and UCS exposure times. During ‘adolescent retrieval’, the metabolic activities of many brain regions of the cognitive/sensory-motor component, of all brain regions of the modulatory component as well as that of the $Hipp_{caud}$ and Sub_{rost} correlated significantly negative – or by trend negative – with the number of avoidances. Vice versa, the metabolic activities of these brain regions correlated significantly positive – or by trend positive – with the number of escapes.

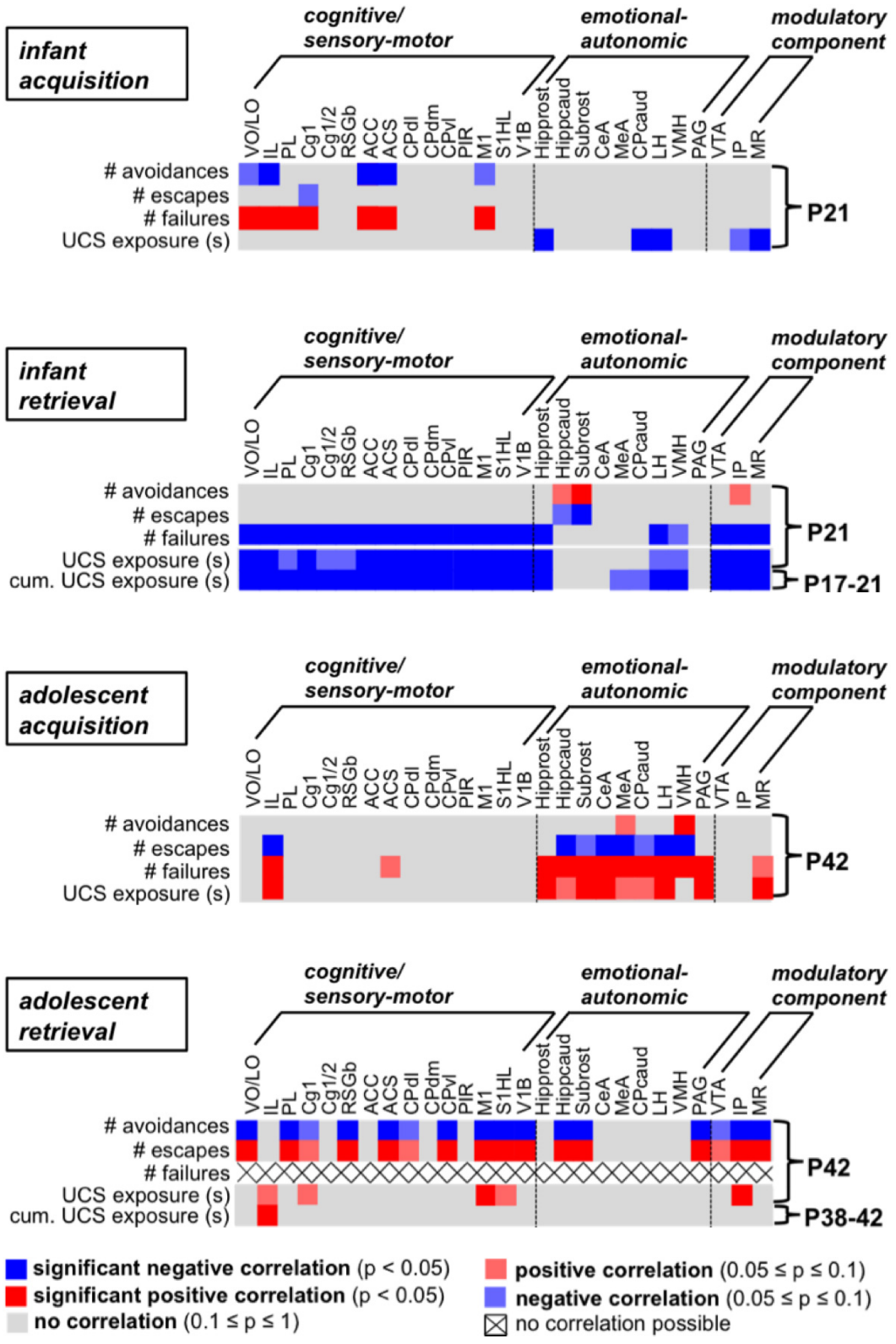


Figure 26: Correlations of behavioral parameters and metabolic activities.

4. Discussion

4.1. Ontogeny of TWA behavior

The study confirmed that infant rats (P17-21) are not able to acquire a sufficient number of avoidances despite massive TWA training. In contrast, adolescent (P38-42) rats perform significantly better than infants (Bauer et al., 1978; Izquierdo et al., 1975; Kudryashova, 2006; Schäble et al., 2007). We recently specified that there is a slow age-dependent increase in the TWA performance in the rat strain we use. Accordingly, the TWA performance of pre-adolescent rats (P24-28) lies in-between that of infants and adolescents; furthermore, adult rats (P80-84) perform significantly better than adolescent rats (Gruss et al., 2010).

4.1.1. The shuttle-box conflict

Provided that there is no sensory or motor impairment, it is usually suggested that the difficulty to acquire and maintain TWA behavior is due to the task complexity. Especially the bi-directional component is rendering this avoidance task difficult because it raises the conflict to shuttle to the opposite chamber where the foot-shock was applied earlier (Gray and McNaughton, 2000; Olton, 1973; Savonenko et al., 1999a).

Infant rats learn avoidance behavior more easily in one-way tasks, which shows that they are able to anticipate the onset of the UCS (Brennan and Barone, 1976; Myslivecek and Hassmannová, 1979; Potash and Ferguson, 1977). The design of one-way avoidance tasks usually implements a safe compartment (dark, target box) and an unsafe compartment (bright, start box). Thus, one-way avoidance learning is not only easier because of the uni-directional component; it also facilitates avoidance learning by additional visual cues whose arrangement utilizes the natural preference of rats for darkness. During TWA training, both compartments of the shuttle-box are illuminated identically, which probably aggravates the existing conflict. In line with this, it has been shown in adult rats: if bi-directional shuttle-box training is performed while simulating a one-way paradigm (constant switching of the box cues according to the location of the animal), the animals perform better than in normal TWA

tasks (Kruger et al., 1969).

We can rule out that our infant rats suffer from motor or sensory impairments as they display a high number of inter-trial changes and often emit non-directed escapes during the initial trials (running and jumping around in a disoriented fashion inside the compartment in response to foot-shock delivery).
Sub-optimal training conditions can also not account for the inability of the infants to learn the TWA task as we have recently shown that stimulus modality modification (tone vs. light) or switch of the diurnal phase during which the training took place (light vs. dark phase) did not had a constant beneficial effect on the performance of infant rats (Rockahr et al., 2010a,b).
Hence, cognitive and/or emotional immaturity obviously renders the solution of the bi-directionality conflict impossible for the infant rats[15].

4.1.2. The infant rats possibly acquire fear

If the training data presented here are analyzed separately, they suggest that the infant rats hardly learned anything. Thus, besides the poor avoidance performance of the infants, there is also no significant difference in the numbers of escapes and failures and consequently also not in the UCS exposure time after five days of TWA training compared to one day of TWA training (Fig. 14).
With regard to this finding, the decrease in the escape latency between P17 and P21 has to be primarily interpreted as a developmental effect. However, we also observed (not quantified) that the initially emitted non-directed escapes step-by-step are converted into directed escapes meaning that infant rats chose the shortest trajectory to the opposite compartment. This could be taken as evidence for a maturation of the

[15] However, it also is not probable, that the infant rats acquire 'learned helplessness'. The situation is consistently escapable for them and they majorly make use of the possibility to escape. Moreover, in 'learned helplessness' paradigms, the uncontrollable/inescapable aversive stimuli produce cognitive deficits. In contrast, in our experiments, infant training facilitates TWA performance during later life. Besides, the amperage applied in our experiments (0.6mA) is sub-threshold for 'learned helplessness' paradigms (up to 1.6 mA; Choi et al., 2010; Maier, 1984).

unconditioned flight reaction. According to Savonenko and colleagues (2003), the acquisition of an appropriate directionality of the escape direction is necessary for the successful learning of the TWA reaction.
The unconditioned escape reaction emitted by the infant rats is different from a genuine escape strategy observable in older rats. Accordingly, adolescent and adult animals that can be considered “escaper” show only exceptionally avoidance reactions. Rather, the CS is a signal for them to become alert and position “in front of the door”. Then, immediately after the UCS-onset, the animals change to the opposite compartment (resulting in escape latencies < 1sec). That type of escape behavior is not observable in infant rats.

From the habituation data, it can be seen that after the first day of TWA training, the infant and adolescent rats behave different in response to shuttle-box exposure. Adolescents stopped their exploration behavior during the second habituation period almost entirely when they were re-exposed to the shuttle-box on day two. This indicates that they remember the context where they experienced the stressful paradigm on the previous day for the first time.
Infants did not show such an inhibition of their exploration behavior during habituation when put back to the shuttle-box on training day two. At first glance, this suggests that the infants did not even acquire fear, which is considered the key prerequisite for the initiation of the instrumental avoidance response in the two-process theory of TWA learning (Miller, 1948; Rescorla and Solomon, 1967; Schlund et al., 2010).

However, this conclusion is not feasible for the following reasons. First, in parallel studies we meanwhile figured out that the infants show a strong stress response after five days of training, which is usually related to the acquisition of fear (Gruss et al., unpublished; Sandi and Pinelo-Nava, 2007; Sullivan et al., 2004). Thus, 1) the percentage of animals that vocalize and 2) the number of distress calls emitted by these individuals and 3) the plasma corticosterone concentration is significantly higher in infant compared to adult rats after five days of TWA training. Therefore, it

seems that if the TWA task cannot be solved, the physiological stress response is aggravated. Nevertheless, this stress response is necessary for the storage of memory contents as blockage of the corticosterone synthesis with Metyrapone dose-dependently abolished the pre-experience effect in infants (Gruss et al., unpublished; Roozendaal et al., 1996).

Second, infant rats can be generally conditioned to fear using classical conditioning paradigms (Pugh and Rudy; 1996). Interestingly, they were most effectively fear-conditioned to a context when CS-UCS pairings occurred within that context (Esmoris-Arranz et al, 2009; Brasser and Spear; 2004). This is in line with our findings demonstrating that the pre-experience effect – depending on the storage of "a memory" – is only produced when CS and UCS are presented contiguous and contingent over five subsequent days of early training (Fig. 3; Gruss et al., 2010).

Finally, coming back to the habituation data, which suggest that the infants do not acquire fear, an interesting finding has been recently demonstrated in mice (Pattwell et al., 2011). These authors showed that early-acquired fear memories undergo behavioral suppression during a specific developmental period. Thus, even if fear was acquired, it was not observable.

Thus, from the behavioral data, we can currently only speculate "what" the infant rats learned. But is it now possible to observe neurobiological substrates of the infant and adolescent training?

4.2. Maturation of functional brain activity in single brain areas

4.2.1. The Septum

Following up this question, we first of all investigated the metabolic activity of the septum in infant and adult rats undergoing TWA learning because both septal subregions have been shown to specifically contribute to TWA performance (Tab. 3; Riedel et al., 2010). The medial septum/diagonal band of Broca (MS/DB) and the lateral septum (LS) play a general role in higher cognitive as well as emotional processes. The MS/DB primarily integrates information about the biological significance of episodes and

events, i.e. contextual information. In contrast, the LS plays an important role in the generation of behavior by enabling the individual to increase interactions with reinforcing stimuli. It is also involved in the processing of the CS-UCS association (Alonso and Köhler, 1984; Calandreau et al., 2007; Jakab and Leranth, 1995; Haghdoost-Yazdi et al., 2009; Lindvall and Stenevi, 1978; Sheehan et al., 2004).

We found that infant rats displayed a lower metabolic activity than adolescent rats in the LS, whereas both age groups showed similar metabolic activities in the MS/DB. These results favored the idea that functional brain activity is still under the process of maturation in brain areas, which are involved in the generation of behavior (LS), whereas brain regions involved in the encoding of memory (MS/DB) are functionally already fully recruited in infant rats[16] (Riedel et al., 2010).
In this article, we argued that the functional immaturity of the LS might be due to not yet fully established dopaminergic and possibly also serotonergic afferents (Antonopoulos et al., 1997; Dinopoulos et al., 1993). Thus, premature processing of motivational (dopamine) and/or aversive (serotonin) information could be a reason for impaired susceptibility of the LS towards reward and reinforcement resulting in an insufficient coordination of adaptive behavioral responses (Cools et al., 2008; Molodtsova, 2008; Olvera-Cortés et al., 2008; Schultz, 2007; Sheehan et al., 2004).

A second important finding of this study was that the LS – but not the MS – was higher activated during TWA acquisition than during TWA retrieval. A similar decrease in energy consumption was also observable in other negatively reinforced tasks (Duncan et al., 1996; Murphy and Feldon,

[16] Cerebral glucose utilization (CGU), glucose transporter (GLUT) expression and the rate constants for glucose transport differentially undergo postnatal maturation up to P30 (Sokoloff et al. 1977). The maturational increases in CGU are related to to GLUT1 (astrocytic) but especially to GLUT3 (neuronal) whose cellular expression is obvioulsy rate limiting during early postnatal development. However, on P21, GLUT3-expression is almost mature and GLUT1-expression has already reached 90% of adult levels (Vanucci et al, 1994).

2001) as well as in "genuine" reward tasks[17] (Tronel and Sara, 2002). Thus, it was suggested that repeated TWA training decreases functional activity not only in the LS but also in a variety of other brain regions and that this could be the result of selection and strengthening of synaptic connections in the process of system consolidation during learning (Frankland and Bontempi, 2003).

As the difference in metabolic activity (acquisition > retrieval) was similar in infant and adolescent rats, it was speculated that processing of the CS-UCS association – known to depend on intact functioning of the LS (Calandreau et al., 2007) – might already be established at this early age. That suggestion would be in line with the above discussed fact that infant animals are able to learn one-way avoidance tasks.

Finally, the lack of decrease in the metabolic activity of the MS/DB after repeated training suggested that – irrespective of the progress in learning – this structure was constantly activated to the same extent. This finding was in line with the results of Toumane and colleagues (1988) who described that cholinergic activity of the septohippocampal pathway immediately after finishing training in a radial maze was similar over a period of nine training days regardless of the increase in performance.

Moreover, the MS/DB was equally activated during exploration of novel and familiar environments as well as retrieval of the TWA task, which was also in accordance with the results of other groups (Wenk et al., 1984[18]).

4.2.2. Further brain areas

As TWA learning is not solely dependent on the activity of a few single brain regions, we, consequently, analyzed the metabolic activity in a number of other brain areas (Tab. 3).

The prediction was that not only brain areas involved in the generation of behavior (like LS) but also those related to association, decision, emotional and reward processes do show a lower metabolic activity in

[17] *c-fos* mapping in the basolateral amygdala during acquisition and retrieval of an odor-reward task

[18] high-affinity choline uptake that the septohippocampal pathway is activated to the same extent by a variety of tasks including active avoidance

infant compared to adolescent rats (infants < adolescents). In contrast, primary sensory and motor regions as well as regions primarily involved in encoding and storage of information (like MS/DB) should show identical task-provoked metabolic activities in infant and adolescent rats (infant = adolescents).
Moreover, metabolic activity should be lower after repeated training in a number of brain areas (acquisition > retrieval) but only in rats that adequately learn the task.

The data collected in this study yielded only limited evidence for these predictions. Rather, infant and adolescent rats displayed very similar effects. In many of the brain areas investigated – i.e. irrespective of their morpho-functional peculiarities – there was a tendency towards or a significant lower metabolic activity in infant rats compared to adolescent rats (infants < adolescents).
In contrast to what was found for the LS, metabolic activity was quasi always[19] similar during acquisition and retrieval (acquisition = retrieval) on the level of single regions.

4.2.3. The VTA and related structures

Interestingly, a few morpho-functionally related brain areas formed an exception of the "rule" that was revealed (infant < adolescent). Thus, the $Cg_{1/2}$, ACS, IP, DR and MR consistently showed a similar metabolic activity in infant and adolescent rats (infant = adolescent). In the VTA, the pattern was even reversed (infant > adolescent).
How could these results be interpreted? It is known that the basal metabolic activity in rats continuously increases over age (Nehlig et al., 1988). Hence, the infant rats "start" from a lower activity level than adolescents when exposed to a specific behavioral challenge. Consequently, when infant and adolescent rats still show this difference after the behavioral challenge, the extent of task-provoked metabolic activity would be similar in both ages. In other words, if the rats display no

[19] except IL in infants, which is regarded a week effect because of the skewness of data distribution (Fig. 20)

differences in metabolic activity after the behavioral challenge, the extent of task-provoked activity would be higher in infants than in adolescents.

Thus, these results could actually indicate that infants[20] display a higher task-provoked activity in serotonergic and dopaminergic brain stem regions and also within two important target regions of these structures, the $Cg_{1/2}$ and ACS (Fig. 27; Ikemoto, 2007; Loughlin and Fallon, 1984; Brog et al., 1993).

The VTA and ACS are involved in TWA learning in adult rats (Carvalho et al. 2009; Murphy and Feldon, 2001; Oades et al., 1987; Reis et al., 2004).
As introduced, we provided pharmaco-behavioral evidence that the dopaminergic system is not yet fully functional in the infant rats[21] (Schäble et al., 2007).
This is possibly due to immature D2-receptor binding in a number of VTA target areas – among them the nucleus accumbens (Johansson et al., 1997). It is also known that feedback projections from the accumbal shell region to the VTA mature late and the earliest time point that their appearance becomes similar to what is seen in adult rats is P21 (Zahm et al., 2001). The accumbal projections are known to primarily exert inhibitory influence on their target regions (Kalivas et al., 1993). Thus, if the VTA is not sufficiently inhibited, a high metabolic activity within this structure and within their target regions is reasonable. Interestingly, it is also known that – although the structural development of the VTA in the rat occurs prior to birth – dopamine transporter density tremendously peaks around P21 reaching 141% of the adult level (Coulter et al., 1996).

In adult rats, it has been shown that an increase in the dopamine signal within the medial PFC is necessary for the formation of a sufficient behavioral strategy during TWA tasks. The fact that the dopaminergic innervation in this region is still incomplete in infant rats could add further

[20] no matter whether they acquire or retrieve a TWA task or whether they experience a novel or familiar environment different from their home cage (see 4.2.3.)
[21] demonstrating that D2-receptor blockade does not yet interfere with the engraving of a memory at early age

evidence for the “dopaminergic hypothesis“ (Kalsbeek et al., 1988; Stark et al., 1999, 2004).

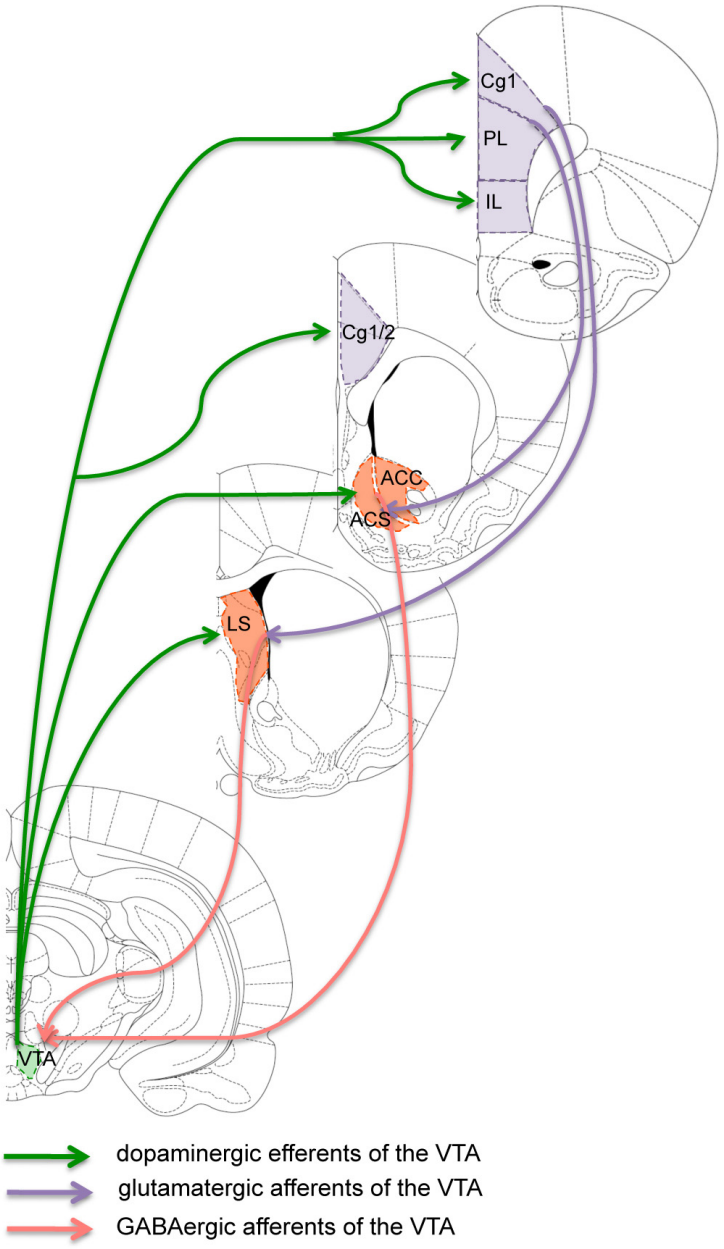


Figure 27: Interconnections between the VTA and their target regions (simplified). It is obvious that the small VTA (covering few neurons, respectively) spreads highly divergent projections to both cortical and subcortical forebrain structures. Thereby, it exerts a profound influence on reward, attention, decision-making and motor performance (Hernandez et al., 2006), i.e. on all processes that are necessary to sufficiently perform a TWA task.

4.2.4. Summary and conclusions

In summary, the data from single region analyses – although with a different lines of arguments – provide further evidence for the hypothesis that immaturity of the dopaminergic system within the mesolimbic circuitry could be one reason for the insufficient formation of the avoidance strategy in infants (Fig. 27).

As the serotonergic system is also involved in TWA learning, late maturation could be supposed for this modulatory system, too[22, 23] (Ensler et al., 1993, Galindo et al., 2008).

The only brain area that was significantly higher activated during acquisition than during retrieval in the adolescent rats was the LS (Riedel et al., 2010). Interestingly, when all measurements of each individual were pooled, respectively, we found that the "overall brain activity" (Fig. 16, suppl. Tab. 2) was significantly higher during acquisition than during retrieval. Thus, the artificial increase in the number of measurements possibly unraveled statistical sub-threshold effects.

The slight group differences are quite feasible because the largest amount of the energy is required for ongoing, basal brain activity. The additional energy burden associated with momentary environmental demands due to environmental challenge can be as little as 1%, and the maximum increase in brain activity is usually estimated around 10 to 20% (Raichle, 2006; Raichle and Mintun, 2006).

[22] The noradrenergic system is the fastest developing modulatory system; noradrenergic innervation of the cortex is already mature around P9 (Levitt and Moore, 1979).

[23] The discussion conducted under 4.2.3. would require a re-interpretation of the MS/DB-data because there was also NO difference between the age groups. However, this is hardly to accomplish because neither the topographical arrangement of the different MS/DB modulatory afferents nor their development are described. It is only known that they usually develop synapses *en passant* along the early maturing cholinergic and GABAergic projection cells of the MS/DB (Risold, 2004).

4.3. Correlated functional activity

Only recently, it was claimed that the cognitive demands, which are necessary for the generation of behavioral output are rather mediated by differential functional networks (Stevens, 2009).

Thus, as a next step, inter-regional correlations of metabolic activities were compiled to see whether differences in the correlated functional activity could account for the respective output of the single behavioral conditions.

4.3.1. Methodological considerations

"Correlated functional activity" has – per definition – to be distinguished from "correlated network activity" or "functional connectivity".

Why? Methods detecting "correlated network activity" in small experimental animals usually analyze local field potentials (LFPs) based on invasive or non-invasive electrode recordings. LFPs reflect concerted synaptic activity of the dendritic arbors of neuronal ensembles (input side of neurons), which are evoked by physiological challenges. These recordings allow a high temporal and spatial resolution during neuronal response monitoring but are hardly to accomplish in freely behaving animals, the more so when the analysis of a variety of brain areas from cortex down to brain stem is required. The analysis of correlated network activity is based on repeated measurements of response signals (basal vs. stimulation) and their statistical correlation.

According to Raichle, neuroimaging signals are also related to the input side of neurons because the local blood flow can be experimentally dissociated from the spiking activity of neurons but not from LFPs (Raichle, 2006; Raichle and Mintun; 2006). Imaging methods like fMRI[24] also analyze the simultaneous activation of two or more brain regions based on repeated *intra*-individual measurements (stimulus on/off). The statistical dependencies between the time-series of the responses are interpreted as "functional connectivity" (Bifone et al., 2010; Pawela et al., 2008).

[24] functional Magnetic Resonance Imaging

In contrast, the 2-FDG method reveals only a single cumulative value (rOD) for the functional (metabolic) activity of every single brain area within a single individual. This rOD covers the entire neuronal and astrocytic activity of a period of 40 to 45 min[25] (Chih et al., 2001).
The slope of the 2-FDG-signal collected over the long period has been described in humans. Accordingly, blood flow and glucose utilization rise immediately after starting a task[26] and remain elevated for more than one hour after its termination (Madsen et al. 1995, 1998).

Thus, we here refer to “correlated functional activity” because this reflects the inter-regional correlations of 2-FDG measurements *between individuals* (Bifone et al., 2010; Nair and Gonzalez-Lima, 1999; Pawela et al., 2008; Soncrant et al., 1986).
Application of the term “metabolic coupling” implements already an interpretive component, as we don’t know whether the statistical relationships between the metabolic activities of brain areas are causative or coincidental. Usually, it is suggested that metabolic coupling is due to increased functional connectivity (Nair and Gonzalez-Lima, 1999; Bifone et al., 2010).
Finally, because the rODs are equal variables, the correlation coefficients do not allow inferences about cause and effect in terms of “activity is going up in one brain area and going down in the other brain area. These inferences about causal interactions require the analysis of “effective connectivity” based on time-series of signals (Sporns, 2007).

4.3.2. PCA revealed three components

PCA allowed us to reduce and systematically arrange the rOD data. Three different sets of brain areas (‘principal components’) explaining 81.32% of the rOD-variability were extracted. According to the algorithmic principle of the PCA, the brain areas (‘factors’) within a single component are closely related to each other. The interpretation of the common underlying

[25] see 2.6. (Addendum)
[26] Wisconsin Card Sorting Task (WCST) that lasted for 20 min

principle, dimension or system is in the hand of the investigator[27].

All subregions of the limbic cortex, a variety of striatal areas and a number of primary sensory cortical areas as well as the primary motor cortex were revealed to be closely related to each other and interpreted to represent the *cognitive/sensory-motor component*.

A variety of areas belonging to the hippocampal formation, amygdala and hypothalamus as well as the PAG were revealed to be closely related to each other and, thus, interpreted to represent the *emotional-autonomic component*. Interestingly, this component also included the rostral hippocampus ($Hipp_{rost}$). This hippocampal subregion – also called dorsal hippocampus – is more involved in cognitive than emotional processing (Bannerman et al., 2004; Kishi et al., 2006; Moser and Moser, 1998).

Three brain stem areas, the VTA, IP and MR, were also revealed to be closely related to each other and interpreted to represent the *modulatory component*. The fact that different regions were not "extracted" by the PCA algorithm means that they contribute less to the variance of the entire data pool. In other words, they "more equally" contribute to the formation of each of the single components. The LS, e.g., processes "cognitive", "emotional" and "modulatory" information and is, thus, not primarily contributing to the formation of a single component.

4.3.3. Correlated functional activity during TWA training

The correlated functional activity of the behavioral conditions was compared based on the trichotomy of principal components.

There are only a few numbers of studies analyzing correlated functional activity similar to what was done in this study.

In awake adult rats, Soncrant and colleagues (1986) described that there is a high positive coupling between adjacent or nearby regions, whereas spatially distant regions are often coupled negatively.

In our study, positively correlated functional activity was also a high among functionally similar and spatially adjacent brain areas in most behavioral conditions (except 'infant acquisition'). But in contrast to what Soncrant et

[27] see 2.8. (Addendum)

al. (1986) described, we also frequently observed positively correlated functional activity between spatially and functionally distant regions like cognitive/sensory-motor and modulatory brain stem areas.
Contrary to these brain areas, the metabolic activities of the emotional and autonomic areas were often not – or even negatively – correlated with those of the other brain areas (Braun et al., 2010).

The lowest degree of positively correlated functional activity was found in the infants trained for one day only (***'infant acquisition'***). In this condition, especially the metabolic activities of the VO/LO, Cg1, PL, IL, ACC and ACS were not significantly correlated, which could give rise for the following speculation.
The prefrontal regions are basically involved in different aspects of executive control meaning that they schedule task operations, which then result in coherent, goal-directed behaviors (Devinsky et al., 1995; Uylings et al., 2003). More precise, the Cg1[28] is primarily involved in executive motor function. The PL[29] is related to working memory, whereas the IL[30] serves a role in viscero-motor control (Gabbott et al., 2005; Heidbreder and Groenewegen, 2003; Vertes, 2006). The VO/LO plays a general role in the encoding of incentive values of associated outcomes and is decisively implicated in emotion processing (Barbas, 2007; Jones and Mishkin, 1972; Schoenbaum and Setlow, 2001). The nucleus accumbens (ACC and ACS) is considered the limbic-motor interface of the brain – it translates motivation into action (Carlezon and Thomas, 2009). If specifically these regions are not yet fully implemented in the brain circuitry on P21 appropriate processing of "cognitive information" and reward during TWA acquisition could be rendered impossible.
As already outlined above, these medial prefrontal and orbitofrontal cortices and the nucleus accumbens have in common that they are innervated by late-maturing dopaminergic fibers that originate in a

[28] Cg1 is the rodent homologue of Brodmann area 24b and the functional homologue of primate frontal eye field and premotor/supplementary motor areas. In rats, it is also known as anterior cingulated cortex (*Acd).*
[29] PL is the rodent homologue of Brodmann area 32
[30] IL is the rodent homologue of Brodmann area 25

topographic pattern from the ventral tegmental area. Moreover, functional dopamine in these structures is required for successful instrumental conditioning (Berger et al., 1991; Cetin et al. 2004; Kalsbeek et al., 1988; Stark et al. 1999, 2004). Thus, the correlated functional activity pattern could provide further evidence for the hypothesis that the dopaminergic system is immature in infant rats.

When rats are trained already for the fifth time on P21 (***'infant retrieval'***), the metabolic activities of the VO/LO, Cg1, PL, IL, ACC and ACS are completely positively correlated with those of the other brain areas – except emotional and autonomic areas. Thus, similar to what has been shown in *Octodon degus* (Gos et al., 2006), the intensive early experience (including handling, separation and TWA training) could have triggered the maturation of modulatory afferents in the preweaning rats, which would then result in increased metabolic coupling.

Interestingly, the patterns of correlated functional activities are very similar during ***'infant retrieval'*** and ***'adolescent acquisition'***. Both conditions do not only display positive coupling within and between the cognitive/sensory-motor and modulatory brain areas but also display a similar extent of uncoupling of the emotional and autonomic areas.
These findings corroborate the idea that the infants are still "under acquisition" during day five of TWA training. Interestingly, we recently showed at the behavioral level, that infant rats require five days of TWA training to induce a significant pre-experience effect, whereas in adolescent rats, one day of TWA training is sufficient to store memory that is of potential benefit (Gruss et al., 2010).

Finally, repeated TWA training leading to an observable avoidance behavior (***'adolescent retrieval'***) was mirrored by a high degree of positively correlated functional activity (except IL, CeA, MeA and VMH). This implies that stronger behavioral challenge increases metabolic coupling, which could be the result of increased functional connectivity.
As introduced, the group of Francisco Gonzalez-Lima revealed a number

of similar findings. These authors described for instance, that functional maturation of frontal cortical regions and their increase in interactions with other brain systems are related to a maturational shift in behavior (Nair et al., 2001). Similarly, they suggested that maturation of the functional connectivity between septal, hippocampal and mesencephalic regions leads to the expression of a certain behavior (Nair and Gonzalez-Lima, 1999).

4.3.4. Correlated functional activity during shuttle-box exposure

In general, correlated functional activity in the "control" conditions (put in quotation marks because they control age for different durations of shuttle-box exposure without TWA training and, thus, reflect a moderate behavioral challenge, too) was relatively similar to what was found in the TWA training conditions.

The most striking difference was seen in the ***'adolescent familiarity'*** condition where the metabolic activity of the CeA and MeA showed a relatively consistent inverse correlation with other brain areas. Repeated shuttle-box exposure is a mild non-stressful challenge for these adolescent rats. Thus, correlated functional activity in the adolescents should approximate basal functional activity.

The emergence of consistent significant negative couplings in the adolescent rats could indicate that there is a more differentiated, interaction among certain areas preparing the brain for a fine-tuned behavioral response. This suggestion would meet a key postulate of the developmental cognitive sciences claiming that age-related cognitive improvements are the result of neural networks becoming more functionally specialized and differentially inter-connected throughout development (Stevens, 2009; Westermann et al., 2006).

In contrast, the regional brain activities in the infant rats are not regulated antidromic. Infant rats probably experience repeated shuttle-box exposure (***'infant familiarity'***) more stressful than adolescents as they have to additionally cope separation from their mother and siblings. In these infants, correlated functional activity is probably not approximating basal

functional activity but rather (still) reflects a serious environmental challenge.

The correlated functional activity during ***'adolescent novelty'*** is very similar to the pattern found during 'adolescent acquisition'. Thus, the novel environment is possibly a similar challenge for adolescent rats like the first TWA training.

What the high number of positive correlations during '***infant novelty'*** means is not clear. It is possibly not reflecting 'metabolic coupling' due to environmental challenge but simply a state of uni-directional regulation of the different regional metabolic activities like an "early default state". Interestingly, very young *Octodon degus* pups (P8) that were socially reared (i.e. together with their parents and siblings) displayed a similar consistent positively correlated functional activity, whereas pups that were considerably disturbed during that age showed a much more "patchy" pattern of correlated functional brain activity (Bock et al., unpublished). However, this proposition is very speculative.

4.3.5. Summary and conclusions

The correlated functional activity patterns differentially reflect age-related behavioral output during TWA acquisition and retrieval. However, it was not revealed that age and training generally increase the degree of metabolic coupling.

The key statements arising from these analyses are that 1) On P21, there are a number of prefronto-cortical regions as well as the nucleus accumbens not fully implemented in the brain circuitry required for TWA acquisition, which could be due to immaturity of the dopaminergic system (Fig. 27). 2) Correlated functional activity in infants suggests that they are still "under acquisition" during their fifth training. 3) Repeated training increases the metabolic coupling in infant and adolescent rats. 4) Age obviously increases the fine regulation of correlated functional activity.

What the interpretation of the inter-regional correlation patterns not allows

is the conclusion that the correlated functional activity is really related to the emitted behavior. Therefore, metabolic activity has to be directly correlated with the behavioral parameters.

4.4. Correlation of behavioral parameters and metabolic activity

4.4.1. Adolescent rats

Starting the discussion with the rats that observably learn the TWA task, it becomes obvious that during ***'adolescent acquisition'***, a high foot-shock exposure correlated positively with the metabolic activities of the emotional and autonomic brain areas. Vice versa, the better escape behavior was already shaped – the lower was the metabolic activity in these areas. Thus, during acquisition, the CeA and related structures metabolically respond in a "dose-dependent" manner: the more aversion the individual experiences, the higher these regions are activated.

This finding implies that the adolescent rats primarily learn to escape on training day one, which is the pre-requisite for the development of a sufficient avoidance strategy (Savonenko et al., 2003). Animals that better learn to escape experience less stress and their emotional and autonomic regions require less energy than those of the rats that learn worse.

Responding of the emotional and autonomic regions is consistent with findings from lesion experiments in adult rats demonstrating that the CeA – as a key component of the emotional-autonomic circuitry – is involved in the acquisition but not in retrieval or maintenance of TWA behavior (Choi et al., 2010; Roozendaal et al., 1993).

During ***'adolescent retrieval'***, the behavioral performance did not correlate with the metabolic activities of emotional and autonomic regions. That means that these regions are always activated to the same extent – no matter whether the rats perform high, intermediate or low during TWA training. Interestingly, van der Borght and colleagues (2005) failed to reveal performance-correlated plasma corticosterone levels and we meanwhile also figured out that the plasma corticosterone in the rat strain we use is not correlated with the avoidance performance (Gruss et al., unpublished).

In accordance with the fourth prediction, we found an inverse correlation of the avoidance performance and the metabolic activities of a number of cognitive/sensory-motor and modulatory brain regions during ‘adolescent retrieval’. This is – together with the correlations revealed for ‘adolescent acquisition’ – consistent with the “memory processing hypothesis” elaborated by McGaugh and Roozendaal (2002). These authors demonstrated in a series of experiments that emotional arousal initially activates the amygdala. The amygdala activation is resulting in an epinephrine-dependent and corticosterone-dependent modulation of the memory, which is finally stored in other brain regions.

Moreover, the inverse correlation of the number of avoidances and the metabolic activity is corroborating the idea that better performance requires less energy consumption in certain brain regions (Brechmann and Scheich, 2005; Sunaert et al., 2000).

4.4.2. Infant rats

Completely different correlation patterns were found during infancy. During ***‘infant acquisition’***, only a few, inconsistently scattered correlations were found indicating that the three morpho-functional units are not specifically involved in the processing of incoming information.

Within ***‘infant retrieval’***, the metabolic activities of the emotional and autonomic areas were not correlated with the behavior. Thus, they were activated independently of the performance also in the infants. However, the majority of cognitive/sensory-motor and modulatory areas as well as that of the rostral hippocampus (the “more cognitive” subregion) was inversely correlated with the number of failures and the UCS exposure time – but never with the numbers of avoidances and failures.

As the tone in the applied TWA paradigm is a hundred percent correlated with the foot-shock exposure (UCS time plus five seconds per trial), the CS exposure is also inversely correlated with these regions (not shown). This could suggests that the brain activity of the infants is simply reflects sensory stimulation or storage of the sensory information. It is probable that this sensory information is already associated up to a certain degree (CS-UCS) and also with the context as it was shown that the infants only

benefit from the early training when CS and UCS are presented contingently and contiguously (Gruss et al., 2010).

4.4.3. Summary and conclusion

The functional activity of certain brain areas is specifically related to different TWA task parameters. Thus, in adolescent rats, activity of the CeA and inter-connected areas is related to the acquisition of the TWA task. In contrast, the activity of cognitive/sensory-motor areas and modulatory brain stem regions is related to storage and successful retrieval of the required memory.

This differential responsiveness of brain areas can be complemented with the model of LeDoux and Gorman (2001). These authors propose that especially the central nucleus of the amygdala (via hypothalamus and brain stem) is involved in generating passive fear reactions whereas the basolateral nucleus of the amygdala (via ventral striatum) is mediating active coping reactions like instrumental responses (Fig. 28).

In the infant rats, the acquisition process is not reflected by specific correlations, whereas activity of a number of cognitive/sensory-motor and modulatory brain stem regions during retrieval reflects failures and UCS (as well as CS) exposure time. Thus, it is possible that the amygdala does not appropriately process incoming information in the infants. Thereby, the correct CS-UCS association, its storage or its implementation in the elaboration of the behavioral plan for the instrumental response, could be prevented (Fig. 28). As we know 1) that infant rats are able to learn a one-way avoidance task (requiring the CS-UCS-association) and 2) that they only benefit from their early experience when CS and UCS are applied contingently and contiguously over five days of training (leading probably to storage of the CS-UCS), the latter possibility – insufficient CS-UCS implementation into a behavioral plan – is the most probable cause for the inferior TWA performance of infant rats.

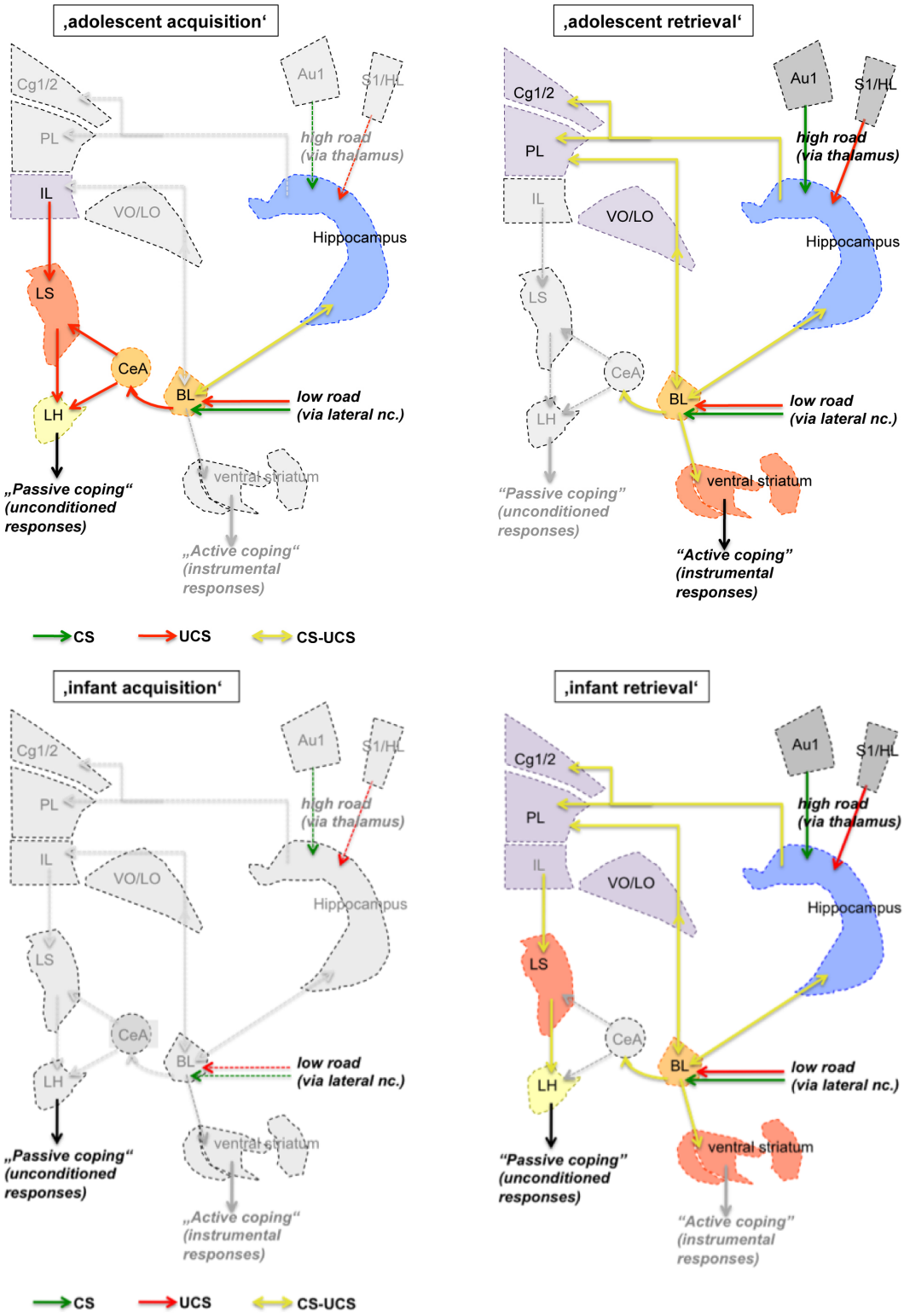


Figure 28: The metabolic activity of different brain areas is differentially correlated to the behavioral output during TWA acquisition and retrieval. The simplified circuits bring together 1) structural connectivity of brain areas (indicated by arrows), 2) functional connectivity of brain areas (indicated by the colors of the arrows) and the resulting behavioral output (passive vs. active coping, after: LeDoux and Gorman, 2001). The light gray-coded brain areas indicate that there is no (or no consistent) correlation of metabolic activity and behavioral parameters. The color-coded brain areas indicate that there is a consistent correlation of metabolic activity and behavioral parameters.

4.5. Implications for future experiments

4.5.1. The acquisition of fear/anxiety

It was proposed that the infant rats acquire fear or anxiety, which is the primary drive for the accelerated instrumental TWA response during adult re-training. However, it is not clear what the extent of the aqcuired fear is and whether it is negatively interfering with the behavioral output during infancy (despite being facilitative during re-training).

We meanwhile have evidence that TWA training during infancy can be of decisive disadvantage. Thus, in *C57BL/6* mice, early TWA training did not only *not* produce a pre-experience effect but was even strongly impeding TWA learning during adulthood (Maas and Braun, 2011). This is very suggestive of being an effect of increased fear-acquisition during infancy (Fig. 29).

If the acquired fear in the rats were adequate and necessary, application of anxiolytics would not improve the infant TWA performance but possibly neutralize the pre-experience effect (Fig. 29). If the acquired fear were too strong in the rats, application of anxiolytics – like benzodiazepines – would increase infant TWA performance and possibly also increase the pre-experience effect[31] (Fernández-Teruel et al., 1991; but see Carvalho et al., 2009). A potent control for this hypothesis would be the *C57BL/6* mice[32].

[31] In my opinion, elaboration of a "dose-response curve" of early-acquired fear and the related potential benefit would be very important in terms of comparative psychobiology. In humans is my impression that personal upbringing and institutional education of children – if they are exclusively based on reward learning – do not in every individual result in an optimal outcome. I have the impression that individuals whose motivation is not so strongly internally driven, could benefit from negative reinforcement. As a matter of course a behavioral outcome in children cannot be motivated by physical aversion. Rather, refusal of different rewards or desires could be used as negative reinforcers. The apprehension to be not rewarded would create a motivation to display a certain behavior. Vice versa, my personal impression is that for individuals whose motivation is stongly internally driven, the additional external pressure that is created by negative reinforcement is disadvantageous. However, this is speculation resulting from non-systematic everyday life "studies".

[32] In addition, in these *C57BL/6* mice the application of Metyrapone would probably restore the avoidance learning because it would block the strong fear memory.

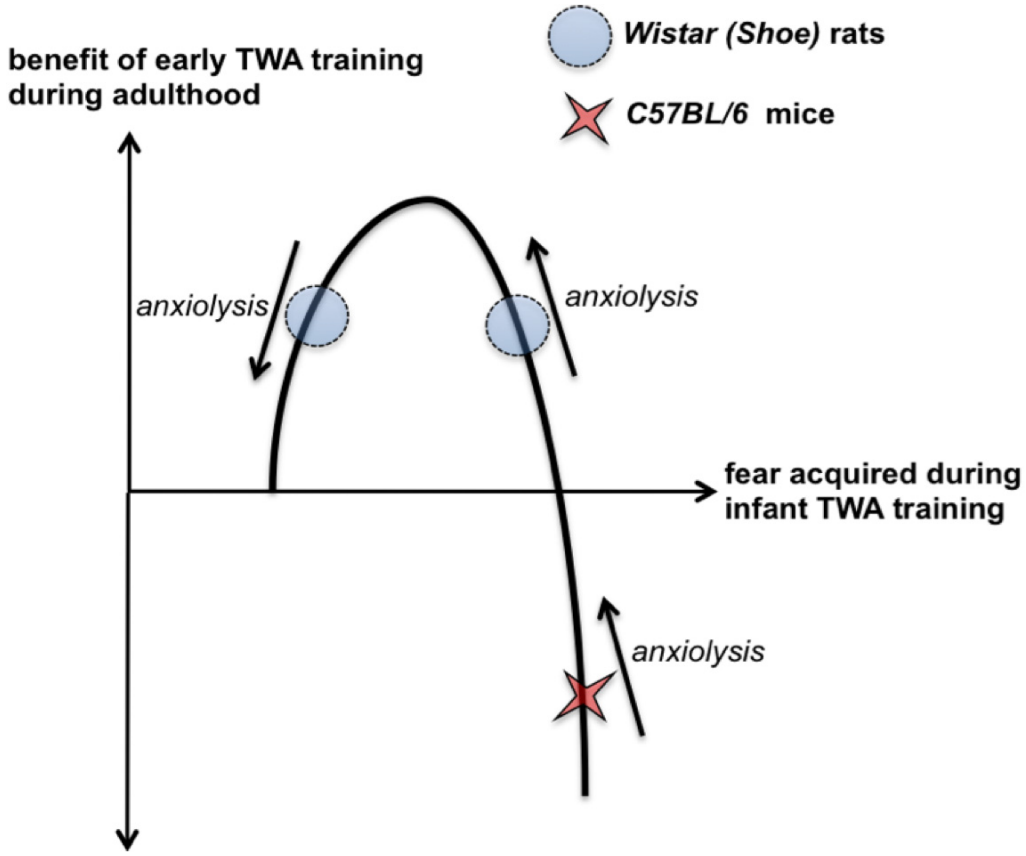


Figure 29: Hypothesized relationship of early-acquired fear and its potential benefit. If the subject acquired too much fear, anxiolysis would improve the potential benefit; if it acquired only little fear, anxiolysis would decrease it.

Accordingly, infant rats selectively bred for high avoidance performance – so called "high avoidance rats" (RHA; Bignami, 1965; Driscoll, 1986) – would learn the TWA task better than normal infants because they are known to be less anxious (Steimer and Driscoll, 2003). Inversely, infant rats selectively bred for low avoidance performance – so called "low avoidance rats" (RLA) – would perform even worse than normal infants and possibly not benefit from their early experience because of a fear overload[33].

Finally, if the early-acquired fear was the primary drive for the accelerated instrumental TWA performance during adult re-training, the pre-experience effect would possibly not – or weaker – occur in a different context and be abolished by lesions of the bed nucleus of the stria terminalis (Sullivan et

[33] Because of the inverse relationship between anxiety and avoidance in these selectively bred rat, I consider the implementation of TWA learning as a model for human anxiety-related disorders or phobias, like it was recently suggested (Choi et al., 2010), not senseful. In humans, high anxiety is ususally related to strong avoidance behavior (Mineka and Zinbarg, 2006) and not vice versa.

al., 2004).

4.5.2. Fear – instrumental response transfer

It has been suggested that different forebrain structures are involved in response suppression during TWA learning in normal adult rats. A huge number of studies have shown, that lesions of the septal subregions and the hippocampus primarily result in increased TWA performance (Gray and McNaughton, 1983; Torras-Garcia et al; 2003). Lesions of the IL – a medial prefrontal region involved in viscero-motor control – also resulted in increased TWA performance (Lacroix et al., 1998). And only recently the group of Joseph LeDoux demonstrated that poor TWA-performing adult rats (not reaching the criterion of 20% after seven training days) immediately after CeA lesion displayed a significant higher number of avoidance responses with further increasing performance over the next five days of training (Choi et al., 2010). The interpretion of these findings was that the "poor performers" had acquired the CS-UCS association but could not generate the behavior. A similar mechanism could account for the infant's inability to emit TWA behavior. Consequently lesions in these areas could add information to this topic.

4.5.3. Maturity stage of the dopaminergic system in infants

It has been described that the successful formation of an avoidance strategy is accompanied by an increase in the dopamine signal within the medial PFC in adult rodents (Stark et al., 1999, 2004). Thus, the same microdialysis experiment could indicate whether this mechanism is intact in the infant rats or not.

If the infants would lack a dopamine increase in the medial PFC comparable to adults, artificial increase of synaptic dopamine by application of Methylphenidate[34] could increase their TWA performance.

Simultaneously specific dopamine receptor agonists (especially DRD2 agonists) could increase the functionality of the dopaminergic system.

[34] Ritalin, a psychostimulant drug, increases the concentration of monoamines (dopamine and norepinephrine) in the synpatic cleft by binding to their transporters, which then results in reuptake inhibition.

External and/or self-stimulation of the VTA (Ilango et al., 2010; Shumake et al., 2010) will possibly not result in an increase in TWA performance because the VTA turned out to be already highly active in the infants.

4.5.4. Implications for the pre-experience effect

The most important question regarding the infant learning is probably, whether the benefit arising from early TWA training is restricted to the avoidance performance or whether it is a genuine improvement of learning and brain capacity[35].

Long-lasting effects of a behavioral challenge on the metabolic capacity can be revealed by cytochrome oxidase (CO) histochemistry[36] (Bruchey and Gonzelaz-Lima, 2008). Like the 2-FDG method, this imaging method allows the simultaneous quantitative imaging of regional brain activity. However – as it covers a longer period (several days) – it reflects the changes in basal activity and is not considered to directly monitor task-provoked changes. Spivey et al. (2011) found, e.g., that repeated maternal separation reduced the CO-activity in the medial PFC and accumbens shell region of these rats. A learning task that is beneficial for the subject possibly increases the CO-activity in a number of brain regions.

From the idealized pre-experience diagram (Fig. 30), it becomes obvious that early training is especially resulting in an accelerated avoidance response between days two and four of adult training. If the early TWA training would really increase the capacity, the pre-experienced rats would learn additional tasks (positively and/or negatively reinforced ones) much

[35] This question is in my opinion also absolutely important with regard to comparative psychobiology. In every-day life, it is freqently observable in children that they display a skill or convincingly demonstrate that they understood an issue much later than it was shown or explained to them. Thus, the lack of instantaneous “output“ should not imply that the children did not learn. However, there is the possibility of capacity-overload – a situation that could be also mimicked in the laboratory rat using the early-learning/pre-experience paradigm.

[36] CO is the rate-limiting enzyme in the mitochondrial transport chain that produces ATP from glucose degradation

better than their naïve littermates within this time frame.

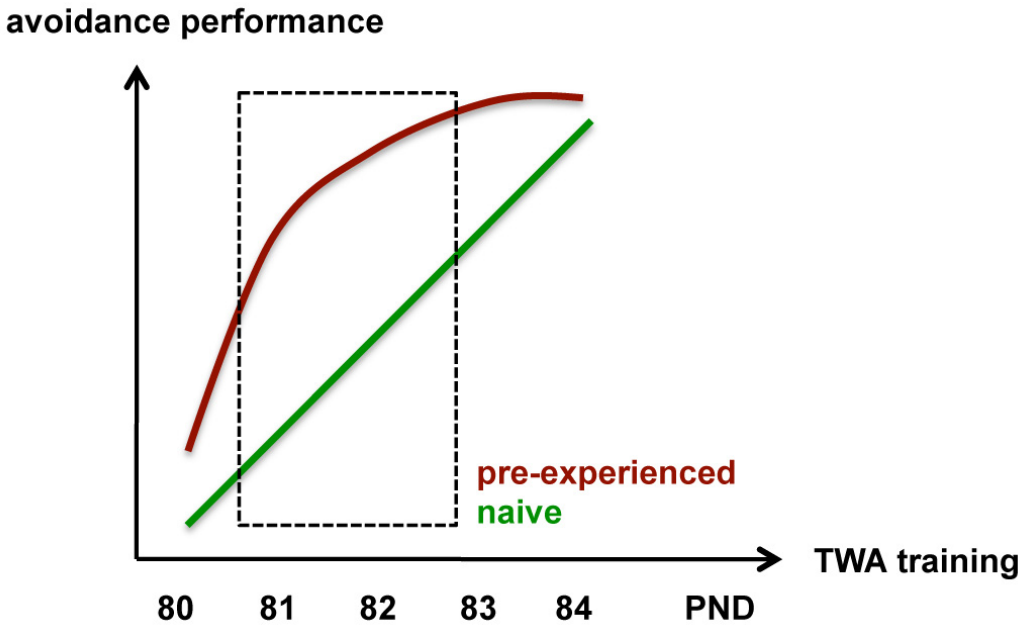


Figure 30: Idealized pre-experience diagram illustrating that especially the time frame between days two and four of adult re-training offers a substrate of increased learning capacity.

Since very recently, short- and long-term changes in functional activity can be elegantly monitored ***within single individuals*** using small animal *in-vivo* imaging methods like single-photon emission computed tomography (SPECT, Heidt et al., 2011). First pilot experiments performed in our group indicate that the resolution (down to 300 µm) is sufficient to map functional activity during TWA learning (Mannewitz et al., 2011). Thus, these analyses could prove evidence that the amygdala of an individual responds during acquisition and, later on, when the task is retrieved, responding of cognitive regions reflect the memory transfer. This differential recruitment of brain areas would not yet be observable in infant rats.

5. Abbreviations

5.1. Anatomical terms

Au1	primary auditory cortex
ACC	accumbens nucleus, core region
ACS	accumbens nucleus, shell region
BL	basolateral amygdaloid nucleus
$BSTl_{dp}$	bed nc. of the stria terminalis, lateral division, dorsal and posterior parts
cc	corpus callosum
CeA	central amygdaloid nucleus
Cg1	cingulate cortex, area 1 (anterior cingulate)
Cg1/2	cingulate cortex, areas 1&2 (posterior cingulate)
CP_{caud}	caudate-putamen, caudal part
CP_{dl}	caudate-putamen, dorsolateral part
CP_{dm}	caudate-putamen, dorsomedial part
CP_{vl}	caudate-putamen, ventrolateral part
CP_{vm}	caudate-putamen, ventromedial part
DR	dorsal raphe nucleus
$Hipp_{caud}$	hippocampus, caudal part
$Hipp_{rost}$	hippocampus, rostral part
LH	lateral hypothalamic area
LS	lateral septum
IC	inferior colliculus
IL	infralimbic cortex
IP	interpeduncular nucleus
M1	primary motor cortex
MeA	medial amygdaloid nucleus
MG	medial geniculate nucleus
MM	mammillary nucleus
MR	median raphe nucleus
MS/DB	medial septal nucleus/Diagonal band of Broca
PAG	periaqueductal gray
PIR	piriform cortex (primary olfactory)
PL	prelimbic cortex
RSGb	retrosplenial granular b cortex
S1HL	primary somato-sensory cortex, hindlimb region
S1BF	primary somato-sensory cortex, barrel field region
Sub_{caud}	subiculum, caudal part
Sub_{rost}	subiculum, caudal part
V1B	primary visual cortex, binocular area
VMH	ventromedial hypothalamic area
VO/LO	ventral/lateral orbitofrontal cortex
VTA	ventral tegmental area

5.2. Other terms

2-DG	^{14}C-2-deoxyglucose
2-FDG	^{14}C-2-Fluoro-2-deoxy-glucose
aa	‘adolescent acquisition’
af	‘adolescent familiarity’
an	‘adolescent novelty’
ar	‘adolescent retrieval’
ia	‘infant acquisition’
if	‘infant familiarity’
in	‘infant novelty’
ir	‘infant retrieval’
mw	molecular weight
rOD	relative optical density
ANLSH	Astrocyte-neuron lactate shuttle hypothesis
CR	conditioned reaction
CS	conditioned stimulus
Da	Dalton
GLUT1	isoform 1 of the glucose transporter
GLUT3	isoform 3 of the glucose transporter
ITI	inter-trial interval
LFPs	local field potentials
NS	neutral stimulus
P	postnatal day
TWA	two-way active avoidance
UR	unconditioned reaction
UCS	unconditioned stimulus

6. References

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7. Addendum

7.1. List of Figures and Tables (chronological order)

CeA, MeA and BL.

p. 49→ Figure 22: All-in-one pair-wise comparisons of metabolic activity in the LH and VMH.

p. 50 → Figure 23: All-in-one pair-wise comparisons of metabolic activity in the VTA, PAG, DR and MR.

p. 51 → Figure 24: All-in-one pair-wise comparisons of metabolic activity in the IC, Au1, S1HL and M1.

p. 53 → Table 6: Principal component analysis.

p. 65 → Figure 25: Inter-regional correlation of metabolic activity.

p. 70 → Figure 26: Correlation of behavior and metabolic activity.

p. 79 → Figure 27: Inter-connectivity of behavior-related forebrain regions innervated by the VTA.

p. 91 → Figure 28: Metabolic activity in infant and adolescent rats is differentially correlated to the behavioral output.

p. 93 → Figure 29: Hypothesized relationship of early-acquired fear and its potential benefit.

p. 96 → Figure 30: Idealized pre-experience diagram

7.2. Supplementary material

Supplementary Table 1: Pair-wise comparisons of behavioral parameters. (1) during the period of 2FDG-accumulation; (2) during the entire training

behavioral condition	Pair-wise comparisons (Wilcoxon rank sum test)				
	# avoidances (1)	cumulative # avoidances (2)	# escapes (1)	cumulative # escapes (2)	# failures (1)	cumulative # failures (2)	UCS exposure time (1)	cumulative UCS exposure time (2)	CS exposure time (1)	cumulative CS exposure time (2)
infant acquisition	**$Chi^2_{(n=17)}=7.039$, $p=0.008$** $Chi^2_{(n=17)}=3.22$, $p=0.070$ **$Chi^2_{(n=17)}=3.83$, $p=0.050$** $Chi^2_{(n=17)}=3.52$, $p=0.060$	**$Chi^2_{(n=17)}=4.904$, $p=0.027$**	$Chi^2_{(n=17)}=5.08$, $p=0.024$ $Chi^2_{(n=17)}=3.36$, $p=0.067$ $Chi^2_{(n=17)}=2.53$, $p=0.111$ $Chi^2_{(n=17)}=2.689$, $p=0.101$	$Chi^2_{(n=17)}=0.593$, $p=0.441$	$Chi^2_{(n=17)}=3.36$, $p=0.065$ $Chi^2_{(n=17)}=0.003$, $p=0.957$ $Chi^2_{(n=17)}=0.06$, $p=0.801$ **$Chi^2_{(n=17)}=3.814$, $p=0.051$**	**$Chi^2_{(n=17)}=5.151$, $p=0.023$**	**$Chi^2_{(n=17)}=8.75$, $p=0.031$** $Chi^2_{(n=17)}=0.93$, $p=0.336$ $Chi^2_{(n=17)}=2.123$, $p=0.145$ **$Chi^2_{(n=17)}=5.333$, $p=0.021$**	**$Chi^2_{(n=17)}=8.333$, $p=0.004$**	**$Chi^2_{(n=17)}=7.253$, $p=0.007$** $Chi^2_{(n=17)}=1.33$, $p=0.248$ $Chi^2_{(n=17)}=2.667$, $p=0.102$ **$Chi^2_{(n=17)}=4.481$, $p=0.034$**	**$Chi^2_{(n=17)}=6.750$, $p=0.009$**
infant retrieval										
adolescent acquisition										
adolescent retrieval										

Supplementary Table 2:
P-values for main factors, post hoc comparisons, mean and SER for:
1) overall metabolic activity (ALL/ALL),
2) regional metabolic activity ('limbic cortex/ALL' … 'sensory/motor/ALL'),
3) metabolic activity in single brain areas ('limbic cortex/VO/LO … sensory/V1B).
Behavioral conditions connected by the same capital letter do not significantly differ from each other regarding their metabolic activity. In contrast, the metabolic activity is significantly different between two behavioral conditions, if they are labeled by different capital letters.

region	area	"condition" (p-value)	post-hoc Tukey for "condition"					rOD mean	± SER	"hemi-sphere" (p-value)
ALL	**ALL**	**<0.0001**	'adolescent acquisition'	A				1.682	0.013	0.645
			'adolescent novelty'	A				1.665	0.014	
			'adolescent familiarity'	A	B			1.626	0.014	
			'adolescent retrieval'		B			1.606	0.011	
			'infant acquisition'			C		1.526	0.013	
			'infant familiarity'			C	D	1.503	0.014	
			'infant novelty'				D	E	1.464	0.014
			'infant retrieval'					E	1.434	0.013
limbic cortex	**ALL**	**<0.0001**	'adolescent acquisition'	A				1.958	0.030	0.751
			'adolescent novelty'	A				1.945	0.032	
			'adolescent familiarity'	A	B			1.844	0.035	
			'adolescent retrieval'	A	B			1.841	0.026	
			'infant acquisition'		B	C		1.779	0.031	
			'infant familiarity'		B	C		1.713	0.031	
			'infant novelty'			C		1.659	0.034	
			'infant retrieval'			C		1.648	0.032	
limbic cortex	**VO/LO**	**0.019**	'adolescent novelty'	A				2.244	0.084	0.688
			'adolescent acquisition'	A	B			2.152	0.078	
			'adolescent familiarity'	A	B			2.106	0.105	
			'infant acquisition'	A	B			2.063	0.073	
			'adolescent retrieval'	A	B			2.038	0.066	
			'infant novelty'	A	B			1.977	0.085	
			'infant familiarity'	A	B			1.957	0.058	
			'infant retrieval'		B			1.809	0.074	
limbic cortex	**IL**	**<0.0001**	'adolescent acquisition'	A				1.566	0.032	0.834
			'adolescent retrieval'	A				1.511	0.024	
			'adolescent novelty'	A				1.503	0.017	
			'infant acquisition'	A	B			1.463	0.054	
			'adolescent familiarity'	A	B	C		1.459	0.028	
			'infant familiarity'		B	C	D	1.329	0.028	
			'infant retrieval'			C	D	1.311	0.038	
			'infant novelty'				D	1.270	0.028	
limbic cortex	**PL**	**<0.0001**	'adolescent acquisition'	A				1.926	0.050	0.673
			'adolescent novelty'	A	B			1.879	0.035	
			'adolescent retrieval'	A	B	C		1.819	0.046	

			‘adolescent familiarity’	A	B	C	D	1.779	0.051	
			‘infant acquisition’	A	B	C	D	1.739	0.057	
			‘infant familiarity’		B	C	D	1.646	0.038	
			‘infant retrieval’			C	D	1.600	0.062	
			‘infant novelty’				D	1.569	0.054	
limbic cortex	**Cg1**	**<0.0001**	‘adolescent acquisition’	A				2.048	0.061	0.964
			‘adolescent novelty’	A	B			2.008	0.045	
			‘adolescent retrieval’	A	B	C		1.918	0.055	
			‘adolescent familiarity’	A	B	C		1.895	0.067	
			‘infant acquisition’	A	B	C		1.822	0.048	
			‘infant familiarity’		B	C		1.748	0.044	
			‘infant novelty’			C		1.718	0.063	
			‘infant retrieval’			C		1.713	0.069	
limbic cortex	**Cg1/2**	0.133	‘adolescent novelty’	A				1.947	0.052	0.905
			‘adolescent acquisition’	A				1.944	0.052	
			‘adolescent familiarity’	A				1.839	0.070	
			‘adolescent retrieval’	A				1.826	0.053	
			‘infant acquisition’	A				1.786	0.076	
			‘infant familiarity’	A				1.767	0.064	
			‘infant novelty’	A				1.729	0.080	
			‘infant retrieval’	A				1.717	0.079	
limbic cortex	**RSGb**	**0.0002**	‘adolescent acquisition’	A				2.114	0.064	0.858
			‘adolescent novelty’	A				2.087	0.065	
			‘adolescent familiarity’	A	B			1.986	0.081	
			‘adolescent retrieval’	A	B			1.934	0.061	
			‘infant familiarity’	A	B			1.828	0.084	
			‘infant acquisition’	A	B			1.799	0.071	
			‘infant retrieval’		B			1.738	0.080	
			‘infant novelty’		B			1.693	0.082	
hippocampal formation	**ALL**	**<0.0001**	‘adolescent acquisition’	A				1.564	0.020	0.237
			‘adolescent novelty’	A				1.532	0.021	
			‘adolescent retrieval’	A				1.501	0.016	
			‘adolescent familiarity’	A	B			1.480	0.016	
			‘infant acquisition’		B	C		1.410	0.023	
			‘infant familiarity’			C		1.368	0.021	
			‘infant novelty’			C		1.342	0.020	
			‘infant retrieval’			C		1.331	0.023	
hippocampal formation	**MS/DB**	0.0645	‘infant acquisition’	A				1.667	0.048	0.451
			‘adolescent acquisition’	A				1.644	0.043	
			‘adolescent novelty’	A				1.585	0.043	
			‘adolescent retrieval’	A				1.582	0.032	
			‘infant retrieval’	A				1.532	0.058	
			‘infant familiarity’	A				1.527	0.044	
			‘infant novelty’	A				1.520	0.047	
			‘adolescent familiarity’	A				1.488	0.030	
hippocampal formation	**$Hipp_{rost}$**	**<0.0001**	‘adolescent acquisition’	A				1.473	0.023	0.741
			‘adolescent novelty’	A				1.434	0.035	

			‘adolescent familiarity’	A	B		1.422	0.017	
			‘adolescent retrieval’	A	B		1.403	0.019	
			‘infant acquisition’		B	C	1.282	0.039	
			‘infant retrieval’			C	1.256	0.035	
			‘infant familiarity’			C	1.240	0.046	
			‘infant novelty’			C	1.181	0.033	
hippocampal formation	**$Hipp_{caud}$**	**0.0015**	‘adolescent acquisition’	A			1.387	0.030	0.729
			‘adolescent retrieval’	A			1.374	0.021	
			‘adolescent familiarity’	A	B		1.347	0.023	
			‘adolescent novelty’	A	B		1.339	0.024	
			‘infant acquisition’	A	B		1.302	0.039	
			‘infant familiarity’	A	B		1.283	0.027	
			‘infant novelty’	A	B		1.265	0.031	
			‘infant retrieval’		B		1.220	0.032	
hippocampal	**Sub_{rost}**	**<0.0001**	‘adolescent acquisition’	A			1.665	0.045	0.092
			‘adolescent novelty’	A			1.652	0.038	
			‘adolescent retrieval’	A			1.593	0.031	
			‘adolescent familiarity’	A			1.580	0.028	
			‘infant acquisition’		B		1.384	0.047	
			‘infant novelty’		B		1.360	0.035	
			‘infant familiarity’		B		1.352	0.039	
			‘infant retrieval’		B		1.276	0.047	
hippocampal formation	**Sub_{caud}**	**<0.0001**	‘adolescent acquisition’	A			1.649	0.042	0.998
			‘adolescent novelty’	A			1.649	0.028	
			‘adolescent familiarity’	A	B		1.563	0.044	
			‘adolescent retrieval’	A	B		1.553	0.036	
			‘infant familiarity’		B	C	1.437	0.033	
			‘infant acquisition’		B	C	1.416	0.036	
			‘infant novelty’			C	1.385	0.027	
			‘infant retrieval’			C	1.371	0.038	
amygdala	**ALL**	**<0.0001**	‘adolescent acquisition’	A			1.253	0.032	0.643
			‘adolescent familiarity’	A	B		1.231	0.033	
			‘adolescent retrieval’	A	B		1.227	0.026	
			‘adolescent novelty’	A	B		1.220	0.038	
			‘infant acquisition’	A	B	C	1.116	0.036	
			‘infant familiarity’		B	C	1.097	0.038	
			‘infant novelty’		B	C	1.088	0.037	
			‘infant retrieval’			C	1.052	0.030	
amygdala	**$BSTI_{dp}$**	**<0.0001**	‘adolescent retrieval’	A			1.096	0.021	0.959
			‘adolescent acquisition’	A			1.090	0.013	
			‘adolescent familiarity’	A			1.069	0.013	
			‘adolescent novelty’	A			1.058	0.011	
			‘infant acquisition’		B		0.951	0.024	
			‘infant retrieval’		B		0.927	0.027	
			‘infant familiarity’		B		0.915	0.027	
			‘infant novelty’		B		0.880	0.027	

amygdala	**CeA**	**<0.0001**	‘adolescent acquisition’	A				1.123	0.021	0.119
			‘adolescent retrieval’	A				1.117	0.011	
			‘adolescent familiarity’	A				1.107	0.017	
			‘adolescent novelty’	A	B			1.063	0.022	
			‘infant familiarity’		B	C		0.979	0.027	
			‘infant acquisition’		B	C		0.975	0.041	
			‘infant novelty’		B	C		0.969	0.029	
			‘infant retrieval’			C		0.935	0.030	
amygdala	**MeA**	**<0.0001**	‘adolescent acquisition’	A				1.127	0.027	0.817
			‘adolescent retrieval’	A				1.117	0.013	
			‘adolescent familiarity’	A				1.094	0.015	
			‘adolescent novelty’	A	B			1.044	0.018	
			‘infant familiarity’		B			0.973	0.032	
			‘infant acquisition’		B			0.972	0.026	
			‘infant novelty’		B			0.970	0.031	
			‘infant retrieval’		B			0.946	0.022	
amygdala	**BL**	**0.001**	‘adolescent novelty’	A				1.715	0.046	0.706
			‘adolescent acquisition’	A				1.671	0.047	
			‘adolescent familiarity’	A				1.652	0.044	
			‘adolescent retrieval’	A	B			1.578	0.027	
			‘infant acquisition’	A	B			1.565	0.056	
			‘infant novelty’	A	B			1.533	0.064	
			‘infant familiarity’	A	B			1.520	0.059	
			‘infant retrieval’		B			1.399	0.045	
striatum	**ALL**	**<0.0001**	‘adolescent acquisition’	A				1.678	0.020	0.812
			‘adolescent novelty’	A				1.676	0.024	
			‘adolescent familiarity’	A	B			1.621	0.024	
			‘adolescent retrieval’	A	B			1.591	0.017	
			‘infant acquisition’		B	C		1.548	0.024	
			‘infant familiarity’			C	D	1.469	0.021	
			‘infant novelty’				D	1.452	0.023	
			‘infant retrieval’				D	1.426	0.022	
striatum	**ACC**	**0.0002**	‘adolescent acquisition’	A				1.712	0.048	0.985
			‘infant acquisition’	A	B			1.682	0.063	
			‘adolescent novelty’	A	B			1.675	0.026	
			‘adolescent familiarity’	A	B			1.634	0.048	
			‘adolescent retrieval’	A	B			1.627	0.037	
			‘infant novelty’		B			1.489	0.046	
			‘infant retrieval’		B			1.476	0.049	
			‘infant familiarity’		B			1.472	0.032	
striatum	**ACS**	**0.0245**	‘infant acquisition’	A				1.692	0.065	0.922
			‘adolescent acquisition’	A				1.629	0.058	
			‘adolescent novelty’	A				1.588	0.047	
			‘adolescent retrieval’	A				1.551	0.044	
			‘adolescent familiarity’	A				1.523	0.058	
			‘infant novelty’	A				1.491	0.051	
			‘infant retrieval’	A				1.458	0.052	

			‘infant familiarity’	A				1.450	0.040	
striatum	**LS**	**<0.0001**	‘adolescent acquisition’	A				1.376	0.030	0.783
			‘infant acquisition’	A	B			1.310	0.029	
			‘adolescent retrieval’	A	B	C		1.285	0.015	
			‘adolescent novelty’		B	C	D	1.237	0.022	
			‘adolescent familiarity’		B	C	D	E	1.215	0.020
			‘infant retrieval’			C	D	E	1.186	0.029
			‘infant novelty’				D	E	1.130	0.022
			‘infant familiarity’					E	1.117	0.022
striatum	**CP_{dl}**	**<0.0001**	‘adolescent novelty’	A				1.997	0.043	0.957
			‘adolescent familiarity’	A				1.907	0.062	
			‘adolescent acquisition’	A				1.905	0.043	
			‘adolescent retrieval’	A	B			1.804	0.044	
			‘infant familiarity’	A	B			1.737	0.047	
			‘infant acquisition’		B			1.651	0.061	
			‘infant novelty’		B			1.643	0.064	
			‘infant retrieval’		B			1.626	0.066	
striatum	**CP_{dm}**	**<0.0001**	‘adolescent acquisition’	A				1.842	0.036	0.829
			‘adolescent novelty’	A				1.828	0.031	
			‘adolescent familiarity’	A	B			1.705	0.045	
			‘adolescent retrieval’	A	B			1.698	0.030	
			‘infant acquisition’	A	B			1.636	0.068	
			‘infant familiarity’		B			1.546	0.040	
			‘infant novelty’		B			1.531	0.064	
			‘infant retrieval’		B			1.526	0.063	
striatum	**CP_{vl}**	**<0.0001**	‘adolescent novelty’	A				1.768	0.034	0.377
			‘adolescent familiarity’	A	B			1.755	0.048	
			‘adolescent acquisition’	A	B	C		1.654	0.026	
			‘adolescent retrieval’	A	B	C		1.636	0.034	
			‘infant familiarity’		B	C	D	1.557	0.031	
			‘infant novelty’			C	D	1.486	0.051	
			‘infant retrieval’				D	1.403	0.049	
			‘infant acquisition’				D	1.377	0.054	
striatum	**CP_{vm}**	**0.0002**	‘adolescent novelty’	A				1.850	0.041	0.915
			‘adolescent acquisition’	A	B			1.808	0.045	
			‘adolescent familiarity’	A	B			1.770	0.057	
			‘adolescent retrieval’	A	B	C		1.694	0.035	
			‘infant acquisition’	A	B	C		1.648	0.070	
			‘infant novelty’		B	C		1.601	0.063	
			‘infant familiarity’		B	C		1.580	0.039	
			‘infant retrieval’			C		1.510	0.065	
striatum	**CP_{caud}**	**<0.0001**	‘adolescent acquisition’	A				1.501	0.028	0.745
			‘adolescent novelty’	A	B			1.463	0.036	
			‘adolescent familiarity’	A	B			1.462	0.022	
			‘adolescent retrieval’	A	B			1.432	0.021	
			‘infant acquisition’	A	B	C		1.390	0.053	
			‘infant familiarity’		B	C	D	1.297	0.036	

			‘infant novelty’			C	D	1.247	0.044	
			‘infant retrieval’				D	1.222	0.024	
hypothalamus	**ALL**	**0.001**	‘adolescent acquisition’	A				1.590	0.067	0.901
			‘adolescent novelty’	A	B			1.511	0.072	
			‘adolescent retrieval’	A	B			1.501	0.049	
			‘adolescent familiarity’	A	B			1.500	0.058	
			‘infant acquisition’	A	B			1.392	0.055	
			‘infant familiarity’	A	B			1.376	0.067	
			‘infant novelty’		B			1.324	0.061	
			‘infant retrieval’		B			1.322	0.057	
hypothalamus	**LH**	**<0.0001**	‘adolescent acquisition’	A				1.418	0.030	0.975
			‘adolescent retrieval’	A	B			1.371	0.020	
			‘adolescent familiarity’	A	B	C		1.342	0.017	
			‘adolescent novelty’	A	B	C	D	1.327	0.027	
			‘infant acquisition’		B	C	D	1.290	0.035	
			‘infant familiarity’		B	C	D	1.245	0.034	
			‘infant retrieval’			C	D	1.221	0.028	
			‘infant novelty’				D	1.204	0.033	
hypothalamus	**VMH**	**0.0001**	‘adolescent retrieval’	A				1.186	0.012	0.193
			‘adolescent acquisition’	A				1.171	0.024	
			‘adolescent familiarity’	A				1.168	0.014	
			‘adolescent novelty’		B			1.051	0.020	
			‘infant acquisition’		B	C		1.006	0.017	
			‘infant retrieval’		B	C		0.984	0.023	
			‘infant familiarity’		B	C		0.980	0.037	
			‘infant novelty’			C		0.933	0.032	
hypothalamus	**MM**	**0.0013**	‘adolescent acquisition’	A				2.180	0.086	0.842
			‘adolescent novelty’	A	B			2.154	0.056	
			‘adolescent familiarity’	A	B	C		1.991	0.080	
			‘adolescent retrieval’	A	B	C		1.946	0.059	
			‘infant familiarity’	A	B	C		1.904	0.076	
			‘infant acquisition’	A	B	C		1.881	0.059	
			‘infant novelty’		B	C		1.834	0.084	
			‘infant retrieval’			C		1.763	0.089	
brain stem	**ALL**	0.06	‘adolescent acquisition’	A				1.542	0.027	0.718
			‘infant familiarity’	A	B			1.487	0.031	
			‘adolescent retrieval’	A	B			1.482	0.024	
			‘adolescent familiarity’	A	B			1.480	0.025	
			‘infant acquisition’	A	B			1.479	0.029	
			‘adolescent novelty’	A	B			1.462	0.025	
			‘infant retrieval’	A	B			1.433	0.027	
			‘infant novelty’		B			1.425	0.029	
brain stem	**VTA**	**0.0002**	‘infant familiarity’	A				1.400	0.029	0.899
			‘infant acquisition’	A				1.382	0.026	
			‘infant retrieval’	A	B			1.374	0.038	
			‘infant novelty’	A	B	C		1.336	0.026	
			‘adolescent acquisition’	A	B	C		1.312	0.027	

			‘adolescent familiarity’	A	B	C			1.297	0.025	
			‘adolescent retrieval’		B	C			1.265	0.021	
			‘adolescent novelty’			C			1.232	0.018	
brain stem	**IP**	0.452	‘infant familiarity’	A					1.717	0.053	0.887
			‘adolescent acquisition’	A					1.671	0.054	
			‘infant acquisition’	A					1.655	0.053	
			‘adolescent familiarity’	A					1.652	0.051	
			‘adolescent retrieval’	A					1.640	0.046	
			‘adolescent novelty’	A					1.631	0.042	
			‘infant novelty’	A					1.607	0.059	
			‘infant retrieval’	A					1.527	0.053	
brain stem	**PAG**	**<0.0001**	‘adolescent acquisition’	A					1.358	0.022	0.601
			‘adolescent retrieval’	A					1.342	0.022	
			‘adolescent familiarity’	A					1.303	0.025	
			‘adolescent novelty’	A	B				1.255	0.018	
			‘infant acquisition’		B	C			1.184	0.031	
			‘infant retrieval’		B	C			1.152	0.025	
			‘infant familiarity’			C			1.120	0.025	
			‘infant novelty’			C			1.095	0.012	
brain stem	**DR**	0.441	‘adolescent acquisition’	A					1.575	0.042	0.686
			‘adolescent novelty’	A					1.542	0.033	
			‘infant familiarity’	A					1.530	0.039	
			‘adolescent familiarity’	A					1.497	0.041	
			‘infant acquisition’	A					1.486	0.055	
			‘adolescent retrieval’	A					1.476	0.036	
			‘infant novelty’	A					1.460	0.042	
			‘infant retrieval’	A					1.452	0.044	
brain stem	**MR**	0.618	‘adolescent acquisition’	A					1.792	0.062	0.742
			‘infant acquisition’	A					1.691	0.066	
			‘adolescent retrieval’	A					1.688	0.050	
			‘adolescent novelty’	A					1.676	0.028	
			‘infant familiarity’	A					1.667	0.046	
			‘infant retrieval’	A					1.660	0.058	
			‘adolescent familiarity’	A					1.650	0.050	
			‘infant novelty’	A					1.628	0.066	
sensory	**ALL**	**<0.0001**	‘adolescent novelty’	A					1.934	0.019	0.774
			‘adolescent familiarity’	A	B				1.896	0.025	
			‘adolescent acquisition’	A	B				1.893	0.021	
			‘adolescent retrieval’		B	C			1.819	0.021	
			‘infant familiarity’			C	D		1.726	0.029	
			‘infant acquisition’				D	E	1.674	0.024	
			‘infant novelty’				D	E	1.671	0.029	
			‘infant retrieval’					E	1.583	0.027	
sensory	**PIR**	**<0.0001**	‘adolescent novelty’	A					1.855	0.037	0.773
			‘adolescent acquisition’	A					1.807	0.042	
			‘adolescent familiarity’	A					1.799	0.052	
			‘adolescent retrieval’	A					1.782	0.041	

			‘infant familiarity’		B			1.542	0.042	
			‘infant novelty’		B			1.534	0.059	
			‘infant acquisition’		B			1.518	0.053	
			‘infant retrieval’		B			1.493	0.055	
motor	**M1**	**0.0058**	‘adolescent novelty’	A				1.992	0.047	0.608
			‘adolescent familiarity’	A				1.957	0.075	
			‘adolescent acquisition’	A	B			1.902	0.058	
			‘adolescent retrieval’	A	B			1.838	0.047	
			‘infant acquisition’	A	B			1.792	0.067	
			‘infant familiarity’	A	B			1.789	0.050	
			‘infant novelty’	A	B			1.779	0.057	
			‘infant retrieval’		B			1.656	0.068	
sensory	**S1HL**	**0.0001**	‘adolescent novelty’	A				1.885	0.043	0.789
			‘adolescent acquisition’	A				1.870	0.044	
			‘adolescent familiarity’	A	B			1.828	0.057	
			‘adolescent retrieval’	A	B	C		1.764	0.049	
			‘infant familiarity’	A	B	C		1.697	0.066	
			‘infant acquisition’	A	B	C		1.693	0.060	
			‘infant retrieval’		B	C		1.590	0.058	
			‘infant novelty’			C		1.561	0.062	
sensory	**S1BF**	**<0.0001**	‘adolescent novelty’	A				1.970	0.062	0.886
			‘adolescent familiarity’	A	B			1.899	0.048	
			‘adolescent acquisition’	A	B	C		1.853	0.042	
			‘adolescent retrieval’	A	B	C		1.788	0.049	
			‘infant familiarity’	A	B	C		1.718	0.060	
			‘infant novelty’		B	C		1.690	0.097	
			‘infant acquisition’			C	D	1.603	0.056	
			‘infant retrieval’				D	1.413	0.042	
sensory	**Au1**	**<0.0001**	‘adolescent novelty’	A				1.914	0.052	0.395
			‘adolescent familiarity’	A				1.864	0.045	
			‘adolescent acquisition’	A				1.851	0.050	
			‘adolescent retrieval’	A	B			1.799	0.057	
			‘infant familiarity’		B	C		1.610	0.042	
			‘infant novelty’			C		1.576	0.045	
			‘infant acquisition’			C		1.571	0.050	
			‘infant retrieval’			C		1.449	0.040	
sensory	**MG**	**<0.0001**	‘adolescent novelty’	A				1.773	0.049	0.879
			‘adolescent acquisition’	A				1.732	0.047	
			‘adolescent familiarity’	A				1.712	0.056	
			‘adolescent retrieval’	A				1.674	0.048	
			‘infant familiarity’	A	B			1.569	0.032	
			‘infant novelty’		B			1.471	0.038	
			‘infant acquisition’		B			1.471	0.034	
			‘infant retrieval’		B			1.470	0.045	
sensory	**IC**	0.288	‘adolescent familiarity’	A				2.241	0.089	0.77
			‘infant familiarity’	A				2.233	0.123	
			‘adolescent acquisition’	A				2.218	0.075	

			‘adolescent novelty’	A				2.136	0.041	
			‘adolescent retrieval’	A				2.077	0.081	
			‘infant novelty’	A				2.068	0.119	
			‘infant retrieval’	A				1.994	0.113	
			‘infant acquisition’	A				1.966	0.085	
sensory	**V1B**	**0.0001**	‘adolescent novelty’	A				1.948	0.048	0.591
			‘adolescent acquisition’	A	B			1.910	0.055	
			‘adolescent familiarity’	A	B	C		1.863	0.063	
			‘adolescent retrieval’	A	B	C	D	1.831	0.058	
			‘infant acquisition’	A	B	C	D	1.736	0.048	
			‘infant novelty’		B	C	D	1.691	0.055	
			‘infant familiarity’			C	D	1.650	0.049	
			‘infant retrieval’				D	1.601	0.054	

Statement of Originality

This PhD thesis has been performed autonomously with the guidance and advice of my supervisors. To the best of my knowledge and belief, this work contains no material previously written or published by another person except as cited and referenced in the text.
The material presented in this thesis has not been submitted for a degree at this or any other university before.

Magdeburg, April 21st, 2011

Anett Riedel