

Effects of social stress on neurogenesis related genes in rainbow trout

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Abstract

In mammals, adult neurogenesis is a restricted phenomenon, taking place in only two distinct areas of the brain, the subventricular zones and the hippocampus. The rate of adult neurogenesis can be regulated through a multitude of factors, including drugs, hormones, environmental complexity, learning and stress. Social stress is a particularly strong negative modulator of adult hippocampal neurogenesis, an effect that may be mediated through glucocorticoid activity. The neurogenic rate may in turn influence memory, learning and mood.

Teleost fish have a considerably higher rate of adult neurogenesis, with proliferative zones located in all parts of the adult brain. Its regulation is, however, still poorly understood. A recent study demonstrates that cell proliferation in the rainbow trout telencephalon is down-regulated in socially stressed individuals. In the current study, quantitative real-time PCR is used to investigate the effect of social stress on the expression of three genes related to neurogenesis in different parts of the rainbow trout brain. These are the proliferation marker PCNA, the neuronal differentiation factor NeuroD and BDNF, which is involved in stimulation of neurogenesis, survival of new neurons and synaptogenesis.

Our results indicate that social stress is not solely a negative modulator of neurogenesis. We found that expression of NeuroD is up-regulated in hypothalamus of socially subordinate rainbow trout, compared to that of dominant fish and controls. This indicates that a rise in neurogenic rate in hypothalamus is caused by social stress experienced by subordinate individuals. Other brain parts exhibited a contrasting pattern, with enhanced PCNA expression in small fish regardless of treatment in the cerebellum and optic tectum.

In view of the possible U-shaped dose response and/or time-response relationship between stress and brain structural plasticity, a more detailed time-course study is called for to determine the detailed effect of chronic stress on the process of neurogenesis in rainbow trout.

Extended Introduction

Adult Neurogenesis

Neurogenesis is the process of cell division by which new neurons are generated. Most neurons are born during embryonic development and persist throughout life in the adult brain circuit, in contrast to other adult tissues that usually harbor stem cells to maintain homeostatic turnover (Li and Xie, 2005; Weissman et al., 2001). The stability of the neuronal circuits at the cellular level has been thought to be essential for the ongoing information processing, and any loss or addition to that circuitry component could undermine the cognitive process as a whole (Rakic, 1985). Not surprising then was the skepticism following the discovery of adult neurogenesis in the 1960s (Altman and Das, 1965). Much has changed since then with adult mammalian neurogenesis being unambiguously established in the early 90s and since demonstrated in all vertebrates examined, including humans (Cameron et al., 1993; Eriksson et al., 1998; Kaplan and Hinds, 1977; Kuhn et al., 1996; Lois and Alvarezbuyla, 1993; McEwen et al., 1996)

Neurons are continuously born throughout life. In the mammalian brain this predominantly occurs in two regions, the subventricular zone (SVZ) lining the lateral ventricles, where new cells migrate to the olfactory bulb via the rostral migratory stream, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, where new cells migrate into the granule layer (Prickaerts et al., 2004). New neurons are generated from neural stem cells through coordinated steps including cell-fate specification, migration, axonal and dendritic growth and finally synaptic integration into the adult brain. This process is divided into four distinct phases: precursor cell phase, early survivor cell phase, post-mitotic maturation phase and late survival phase (Kempermann et al., 2008). The precursor cell phase serves the expansion of the pool of cells that might differentiate into neurons. Early survivor phase marks the exit from the cell cycle, after which many of the newly generated cells migrate from the proliferation zones reaching their target areas within ten days of formation. After reaching their designated location many of the newborn cells are eliminated through apoptosis. The post-mitotic phase is associated with maturation and formation of functional connections, growth of axons,

dendrites and synaptogenesis. The late survivor phase represents a period of fine tuning and final incorporation into the central nervous system (CNS) (Kempermann et al., 2008). On the basis of cell morphology a set of marker proteins can be identified that define distinguishable steps in the development of new neurons, useful in quantification and progress determination of neurogenesis.

The functional significance of the newborn neurons is still not clear, but one popular hypothesis is that adult neurogenesis might be essential in maintaining normal cognition and memory formation mammals (Abrous and Wojtowicz, 2008; Ge et al., 2008; Perera et al., 2008). This is perhaps not surprising as hippocampus, one of the two sites of mammalian adult neurogenesis, is well known for its importance in memory formation, mood regulation, spatial navigation and endocrine control (Drevets, 2001; Eichenbaum, 1999a, 1999b; Squire, 1992). Hippocampus is for instance one of the first regions of the brain to suffer damage in Alzheimer's disease, with memory problems and disorientation being among the symptoms to appear. Also extensive damage of the hippocampus has been implicated in amnesia (Goodrich-Hunsaker and Hopkins, 2009; Kesner and Goodrich-Hunsaker, 2009). The theory that hippocampal neurogenesis is important in formation of new memories and learning comes from the observation that associative induction of long term potentiation is much easier in new neurons than in mature neurons under the same conditions (Schmidt-Hieber et al., 2004). Gould demonstrated in 1999 (Gould et al., 1999a) that the act of learning itself is associated with increased neuronal survival and integration after neurogenesis. Studies have also shown that X-ray irradiation of hippocampus and chemical inhibition of its neurogenesis both decrease learning ability (Rola et al., 2004; Vitral et al., 2006). Thus, having a higher rate of hippocampal neurogenesis would imply a better cognitive performance and ability to interpret a situation based on previous experience, increasing predictability and reducing perception of stress. Adult neurogenesis is affected by a range of environmental, endocrine and pharmacological factors (Bodnoff et al., 1995; Brezun and Daszuta, 1999; Duman et al., 2001; Huang and Herbert, 2005; Jacobs et al., 2000; Mora et al., 2007) . In particular, physical activity (Eadie et al., 2005; van Praag et al., 1999), environment complexity (Kempermann et al., 1997; Nilsson et al., 1999) and various stressors (Duman

et al., 2001; Fuchs et al., 2006; Gould et al., 1997; Gould et al., 1998; Malberg and Duman, 2003; Mirescu and Gould, 2006; Sapolsky, 2003).

Voluntary physical activity and enhanced environmental complexity elevate the rate of neurogenesis in the dentate gyrus, which, in turn has been found to correlate with improved performance in hippocampal-dependant learning and memory tasks (Gould et al., 1999b; Nilsson et al., 1999; van Praag et al., 1999). On the other hand, stress has been shown to reduce hippocampal neurogenesis (Fuchs and Flügge, 1998; Gould et al., 2000; Joëls et al., 2004), an effect that may in part be caused by elevated glucocorticoid hormone levels. There are high densities of glucocorticoid hormone receptors present in the hippocampus (Sapolsky et al., 1986), and administration of glucocorticoid hormones inhibits hippocampal neurogenesis (Gould and Tanapat, 1999).

Markers of neurogenesis

For analysis and quantification of neurogenesis several marker proteins can be used. These will represent different stages in the neurogenic process.

DNA polymerase δ (pol δ) is necessary for replicating the entire length of a DNA template during S-phase of the cell cycle. It is able to do this in complex with a protein named proliferating cell nuclear antigen (PCNA). PCNA is a DNA clamp, binding the DNA, after which pol δ binds to PCNA, now able to copy an entire template without dissociation. PCNA appears to be a reliable marker of brain cell proliferation in teleosts as it is expressed in growing cells with a proliferative potential, and is absent in quiescent cells (for discussion of the use of PCNA as a proliferation marker in teleost CNS, see Wullimann and Puelles, 1999). Its expression has been shown to overlap very closely with nuclei that have recently incorporated injected bromodeoxyuridine (BrdU), a thymidine analog and S-phase marker (Ekström et al., 2001; Grandel et al., 2006; Villar-Cheda et al., 2006).

After cell proliferation in the CNS, the precursor cell must differentiate into a functional neuron. For this, appropriate determination and differentiation factors are needed to control the maturation of new cells. NeuroD is a basic helix-loop-helix transcription factor found in newly determined neuronal cells, regulating cell cycle withdrawal and early neuronal differentiation (Miyata et al., 1999; Pleasure et al., 2000). It has been suggested that adult hippocampal neurogenesis may be dependent upon NeuroD activity (Miyata 1999), as increase in neurogenesis following excitatory activity, correlates with an increase in NeuroD expression (Deisseroth et al., 2004). NeuroD expression has been studied extensively in postembryonic and adult zebrafish, where it is present in post-mitotic cells, functioning as an early differentiation factor immediately after determination of neuronal fate (Korzh et al., 1998; Mueller and Wullimann, 2002a, 2002b).

Neurotrophic substances stimulate the growth and survival of developing neurons, and also help maintain adult neurons and increase survival of damaged neurons by promoting axon and dendrite growth. Neurotrophic support is an essential component of the signal cascade that controls generation of new neurons. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and has a central role as a stimulant for generation, differentiation and survival of new neurons, as well as guiding newly generated neurons to their proper site of synaptogenesis (Benraiss et al., 2001; Rossi et al., 2006; Sairanen et al., 2005; Scharfman et al., 2005; Zigova et al., 1998). Administration of BDNF into proliferative areas of the brain leads to increased levels of neurogenesis (Scharfman et al., 2005; Zigova et al., 1998), while heterozygotic BDNF-knockout mice display reduced levels of neurogenesis (homozygotic knockout is lethal) (Lee et al., 2002). Interestingly, BDNF levels are downregulated by both acute and chronic stress (Nibuya et al., 1995;1999; Smith et al., 1995), and by glucocorticoid hormones (Smith et al., 1995).

Neurogenesis in teleost fish

There are many remarkable differences between adult neurogenesis in mammals and teleosts, making their comparison very interesting for understanding the evolution of the phenomenon. As previously mentioned, adult neurogenesis in mammals is restricted only to two areas in the brain. In contrast, several such areas have been identified throughout the entire brain in teleost fish, including regions homologous to the ones found in mammalian olfactory bulb and hippocampus (Ekström et al., 2001; Grandel et al., 2006; Hinsch and Zupanc, 2007; Zikopoulos et al., 2000; Zupanc and Horschke, 1995). The rate of proliferation in these areas is at least ten times higher than the neurogenic rate in the adult mammalian brain. In zebrafish (*Danio rerio*), 6000 new cells are generated every half hour, corresponding to 0.06% of the total population of brain cells (Hinsch and Zupanc, 2007), while in the subventricular zone of adult mice only 30.000 new cells are formed every day (Williams et al., 2000). This is about 600 cells per half hour, or 0.0006% of the estimated 110 million cells in the mouse brain. Areas of adult brain cell proliferation have been identified in all teleosts examined including *Apteronotus leptorhynchus* (Zupanc and Horschke, 1995), zebrafish (*Danio rerio*, Grandel et al., 2006; Zupanc et al., 2005), three-spined stickleback (*Gasterosteus aculeatus*, Ekström et al., 2001) and rainbow trout (*Oncorhynchus mykiss*, Lema et al., 2005).

Depending on the brain region, the newly generated cells either remain in the vicinity of the area they were born or migrate to specific target areas in the first 1-2 weeks after generation. In most proliferation zones of the cerebellum, situated within the molecular layers, new cells migrate into the granule cell layers. On the other hand, in the proliferation zones of the optic tectum the new cells remain in the immediate vicinity during their subsequent development and maturation (Ekström et al., 2001; Zupanc et al., 2005). After reaching their final destination, at least 50% of the newly generated cells are eliminated through apoptosis, while the remaining cells can persist for the rest of the fish's life (Grandel et al., 2006; Zikopoulos et al., 2000; Zupanc et al., 2005), the number of long term surviving cells varies greatly with the fish species and brain region. The

continuous generation and high survival rate of cells in the teleost brain leads to its permanent growth throughout life (Kaslin et al., 2008; Zupanc, 1999, 2001), the number of brain cells increasing with age, body weight and body length (Birse et al., 1980; Zupanc and Horschke, 1995). The generation of new cells and the elimination of damaged cells through apoptosis enable teleost fish to have rapid and efficient neuronal regeneration after brain and sensory neuron injuries. Their potential for CNS repair includes both regeneration of axons (Doyle et al., 2001; Matsukawa *et al.*, 2004; Stuermer *et al.*, 1992) and whole neurons (Cameron, 2000; Zupanc and Ott, 1999). In mammals, regeneration of structures in the CNS is highly limited due to the low rate and restricted distribution of neurogenesis as well as necrosis of neurons surrounding the site of brain damage, causing secondary inflammation (Strbian et al., 2009). A potential function has been suggested for the astounding rate of cell proliferation and regeneration potential in teleosts, involving their postembryonic development of motor structures and sensory organs (Zupanc, 2001, 2008; Zupanc and Ott, 1999). The higher rate of neurogenesis in fish might be due to the need of neuronal innervation and control of the increasing number of muscle fibers and sensory cells. While in mammals, growth implies an increase in size and not amount of muscle fibers (Rowe and Goldspink, 1969), in fish both the number of muscle fibers and volume of individual fibers increase with age and body weight (Weatherley and Gill, 1989).

Stress and stress responses

McEwen and Stellar (1993) defined stress as the state where an intrinsic or extrinsic factor, a stressor, poses a real or imagined threat to an animal's homeostasis, where homeostasis refers to the maintenance of a narrow range of vital physiological parameters necessary for survival. An animal's stress response is the collective physiological and behavioral adaptations initiated to re-establish homeostasis.

The stress response is mediated by the stress system, located in the central nervous system (CNS) and periphery (Chrousos, 1998; Chrousos and Gold, 1992). In response to a stressor the stress system integrates neurosensory and endocrinological

signals to modulate behavioral and physiological changes accordingly. Increases in respiration rate, heart rate, blood pressure and glucose metabolism are among the parameters affected by the stress response (Selye and Fortier, 1950). They serve to prepare the animal to better cope with the stressor. The endocrinological stress response in mammals consists of the hypothalamic-pituitary-adrenal, (HPA) axis and the sympathico-adrenomedullary system. The mammalian HPA axis mediates feedback interactions between the hypothalamus, the pituitary gland and the adrenal gland. Control of the stress response is one of the major tasks of the HPA axis. The elements of the mammalian HPA system are the paraventricular nucleus of the hypothalamus that synthesize and secrete corticotrophin-releasing hormone (CRH). CRH in turn stimulates the anterior lobe of the pituitary gland to secrete adrenocorticotrophic hormone (ACTH). Glucocorticoid hormone production and secretion from the adrenal cortex is stimulated by elevated levels of plasma ACTH, and lastly glucocorticoids form a negative feedback loop suppressing CRH and ACTH release in hypothalamus and pituitary gland, respectively. The main glucocorticoid in humans and teleost fishes is cortisol. In teleosts the system is called hypothalamic-pituitary-interrenal (HPI) axis, because fish do not have a distinct adrenal gland. The adrenal cortical tissue is represented by the interrenal cells that secrete glucocorticoids upon ACTH stimulation (Mommensen et al., 1999). Teleost fish also lack a hypothalamic-hypophyseal portal system and CRH control of ACTH release is achieved through direct neuronal innervation. Cortisol mediates its effect through binding to two types of receptors; Type I, mineralocorticoid (MR), and Type II, glucocorticoid receptors (GR) (De Kloet et al., 1985).

Stress and neurogenesis

A few studies have demonstrated that as in mammals, rates of brain cell proliferation in fish can be affected by external factors. Environmental enrichment increases the size of brain structures in rainbow trout, including cerebellum, optic tectum and telencephalon (Kihlslinger and Nevitt, 2006; Marchetti and Nevitt, 2003). Two studies have also demonstrated a change in telencephalic cell proliferation due to

increased environmental complexity (Lema et al., 2005; Von Krogh et al., submitted). Sørensen et. al. (2005) showed that social stress reduces cell proliferation in the telencephalon of socially subordinate rainbow trout. In this study size matched pairs of rainbow trout fought for dominance and were afterwards left to interact for four days. In these pairs, the subordinate fish had reduced cell proliferation in the telencephalon compared to isolated controls. Plasma cortisol analysis of the fish indicated that they were chronically stressed due to the aggressive social interactions after hierarchy formation. The aim of this study was to investigate the effect of social stress on the expression of three genetic markers for cell proliferation and neuronal differentiation; proliferating cell nuclear antigen (PCNA) (Takasi et al., 1981), brain-derived neurotrophic factor (BDNF) (Barde et al., 1985) and Neurogenic differentiation factor (NeuroD) (Chae et al., 2004).

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Effects of social stress on neurogenesis related genes in rainbow trout

Introduction

Adult neurogenesis is an example of a long-held scientific theory being overturned. Early neuroanatomists considered the nervous system to be fixed and incapable of regeneration, but since the pioneering studies of Altman in the early 1960s (Altman and Das, 1965), the process of adult neurogenesis has been unambiguously established, and confirmed in all mammals examined, including humans (Cameron et al., 1993; Eriksson et al., 1998; Kaplan and Hinds, 1977; Kuhn et al., 1996; Lois and Alvarezbuylla, 1993; McEwen et al., 1996). Generation of new neurons in the central nervous system of adult mammals seems to be restricted to two regions; the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus. The functional significance of the adult-born neurons is still not evident, but is being actively investigated in mammals and other vertebrates. The turnover of neurons as a result of neurogenesis and death of old cells through apoptosis seems to be essential in maintaining neural plasticity and normal cognition in mammals (Abrous and Wojtowicz, 2008; Ge et al., 2008; Perera et al., 2008).

In teleost fish, adult neurogenesis can be found in all major brain regions, and takes place at a much higher rate than in mammals (Ekström et al., 2001; Grandel et al., 2006; Zikopoulos et al., 2000; Zupanc et al., 2005; Zupanc and Horschke, 1995). Also, a high degree of long term survival for newborn fish neurons has been demonstrated, where, depending on the species and brain area, up to 50% of the new born cells at any time can persist for the rest of the fish's life (Grandel et al., 2006; Zikopoulos et al., 2000; Zupanc et al., 1996; Zupanc et al., 2005). This contributes to the life-long growth of the fish brain (Birse et al., 1980a; Zupanc and Horschke, 1995), and this pool of new cells are also involved in the fish brain's impressive capacity for regeneration of damaged neuronal tissue (Cameron, 2000; Doyle et al., 2001; Matsukawa et al., 2004; Stuermer et al., 1992; Zupanc and Ott, 1999).

In mammals, the rate of adult neurogenesis can be affected by a range of environmental, endocrine and pharmacological factors (Bodnoff et al., 1995; Brezun and Daszuta, 1999; Duman et al., 2001b; Huang and Herbert, 2005; Jacobs et al., 2000; Mora et al., 2007) including physical activity (Eadie et al., 2005; van Praag et al., 1999), environmental complexity (Kempermann et al., 1997; Nilsson et al., 1999) and various stressors (Duman et al., 2001a; Fuchs et al., 2006; Gould et al., 1997; Gould et al., 1998; Malberg and Duman, 2003; Mirescu and Gould, 2006; Sapolsky, 2003). Social stress appears to be especially potent in reducing neurogenic rate (Blanchard et al., 2001; Gould et al., 1997; Gould et al., 1998), and this reduction is at least in part caused by elevated glucocorticoid hormone levels (Fuchs and Flügge, 1998; Gould et al., 2000; Joëls et al., 2004). Indeed, administration of glucocorticoids inhibits hippocampal neurogenesis (Gould and Tanapat, 1999). The reduced hippocampal neurogenesis seen during social stress is also accompanied by a general behavioural inhibition.

Salmonid fishes are aggressive and territorial and form clear dominance hierarchies in the wild, as well as in experimentally established pairs, where dominant individuals monopolize food resources and initiate almost all aggressive attacks (Höglund et al., 2002; Øverli et al., 1999; Øverli et al., 1998). Holding a subordinate position in such a hierarchy is very stressful and leads to a general behavioral inhibition characterized by reduced feeding, low locomotor activity and suppressed aggressive and reproductive behavior (Abbott and Dill, 1985; Amorim and Almada, 2005; Francis et al., 1993; Huntingford et al., 1993; Oliveira and Almada, 1998; Øverli et al., 1998; Winberg and Nilsson, 1993), as well as activation of the hypothalamus-pituitary-interrenal (HPI) axis, which results in increased cortisol secretion, and chronically increased brain serotonin (5-hydroxytryptamin, 5-HT) metabolism (Doyon et al., 2003; Elofsson et al., 2000; Francis et al., 1993; Höglund et al., 2000; Øverli et al., 1999; Winberg and Nilsson, 1993; Winberg et al., 1992). This is thought to be a passive coping response that serves to avoid aggressive interactions with dominant individuals (Leshner, 1980). Sørensen (2005) demonstrated that socially subordinate rainbow trout have significantly decreased telencephalic cell proliferation compared to control fish.

In the present study we venture to investigate the effect of social stress on the expression of three genetic markers of cell proliferation and neuronal differentiation; proliferating cell nuclear antigen (PCNA) (Takasaki et al., 1981), brain-derived neurotrophic factor (BDNF) (Barde et al., 1985) and Neurogenic differentiation factor (NeuroD) (Chae et al., 2004).

Materials and methods

Experimental animals

The experimental animals used were juvenile rainbow trout, weighing 123.7 ± 6.7 g (N=56), obtained from Valdres Ørretoppdrett in Valdres, Norway. Prior to the experiment, the fish were kept for more than 1 month in 750 liter holding tanks at the aquarium facilities at the University of Oslo. Tanks were supplied with aerated dechlorinated Oslo tap water at 5-7°C and artificial lighting followed a 12 hour light and 12 hour darkness cycle. The fish were fed 1% of their body weight (Ewos pelleted trout food) each day.

Experimental setup and sampling

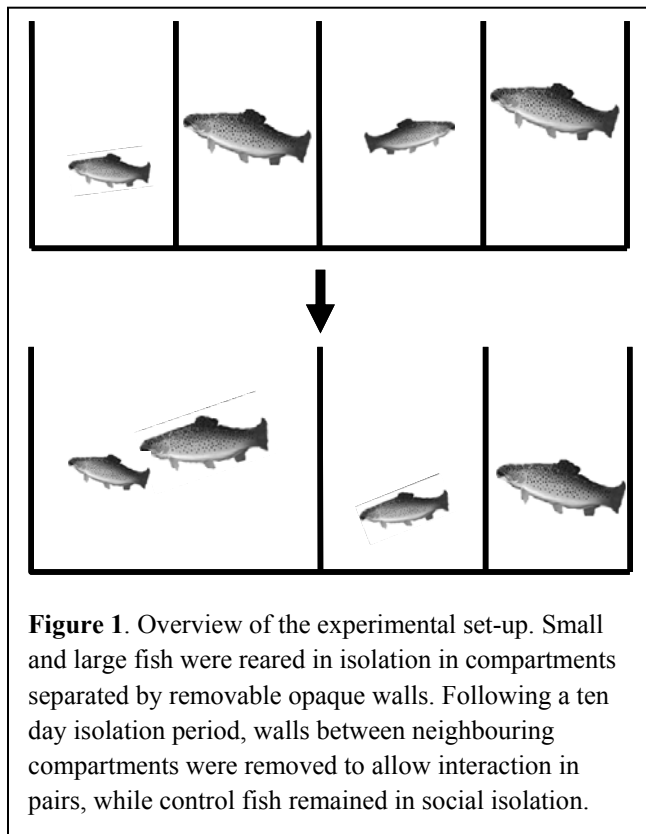
The experiment was conducted in eight 250L glass aquaria, divided into four separate 60 l compartments by removable opaque PVC walls. The bottom and sides of the aquaria were covered on the outside with black plastic film and each compartment was lidded with plastic mesh. The experimental aquaria were continuously supplied with aerated dechlorinated Oslo tap water (5-7°C, 0.25 l/min, 12h/12h light/darkness).

Experimental fish were selected from the holding tanks and anesthetized lightly with MS-222 (Tricaine methanesulfonate, 0.25g/l). Their weights were recorded, after which they were placed in the observation aquaria. The fish were weight-matched in neighboring pairs, with one fish 50-100% heavier than the other. For acclimation, the fish were left isolated for 10 days, during which time they were hand fed daily, 1% of their

body weight (Ewos pelleted trout food). Food items not consumed were removed immediately after feeding.

On day eleven the fish were anesthetized lightly and weighed. On the following day, the walls between 16 randomly selected neighboring compartments were removed allowing the fish to interact and establish a social hierarchy. The remaining fish were kept isolated as controls. Hence, four experimental groups were formed; small isolated controls, large isolated controls, small subordinate fish and large dominant fish

When the walls between adjoining compartments in the aquaria were removed,



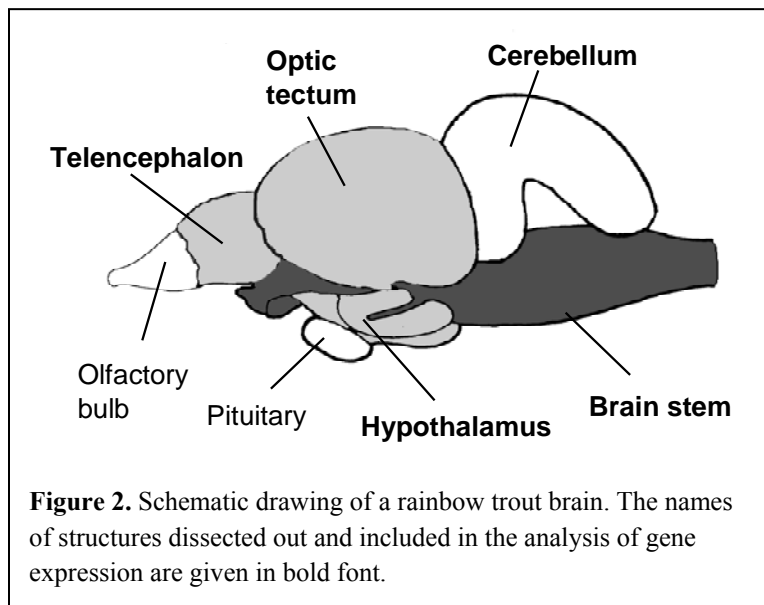
the fish would, after a latency ranging from 30 s to half an hour, start fighting. Aggressive behavior consisted of chasing and biting. Duration of the fights ranged from a few seconds to half an hour, reaching conclusion when one fish retired from further aggression, becoming the subordinate animal. The dominant animal would continue attacking the subordinate with varying intensity and at varying time intervals.

After establishment of the social hierarchy, the fish were left together for a period of four

days. The pairs were filmed for ten min immediately after conclusion of the fight, and ten min at a random time period between 10.00 and 14.00 prior to feeding each day. From these films aggression was quantified, counting the number of successful attacks, which was defined as a bite or hit to the other fish's body. During the time spent in pairs, the

fish received 1% of the pair's total body weight in feed. The controls were fed as in the acclimation part of the experiment.

On day fifteen all fish were rapidly netted and anesthetized, in 1g/l MS-222, fish pairs were taken out simultaneously. The fish were weighed whereupon approximately 1 ml of blood was taken from the caudal vein using a syringe containing a minute amount



of EDTA (ethylenediaminetetraacetic acid, Sigma). The blood was centrifuged at 3000 rpm for 3 min, and the plasma was frozen in liquid nitrogen and stored at -80°C until quantification of cortisol with radioimmunoassay (RIA). The fish were killed by decapitation and the brain was

dissected out and divided into the following parts: telencephalon, hypothalamus, cerebellum, brain stem and optic lobes (Figure 2). The parts were wrapped in aluminum foil and frozen in liquid nitrogen. The samples were stored at -80°C until mRNA extraction.

Molecular techniques

The expression of brain derived neurotrophic factor (BDNF), proliferating cell nuclear antigen (PCNA) and Neurogenic differentiation factor (NeuroD), was quantified with Real-Time PCR in telencephalon, hypothalamus, cerebellum, optic lobes and brain stem. Total RNA from each tissue sample was isolated and treated with DNase. Quality and quantity of total RNA was determined with Nanodrop spectrophotometer and Agilent Bioanalyser, after which cDNA was synthesized using total RNA as template in a reverse

transcriptase reaction. Lastly the cDNA was amplified and quantified with Real-Time PCR using primers for our genes of interest. Primers were previously designed by Ida Beitnes Johansen. Quantity of gene expression was calculated in relation to expression of β -actin, a gene found to be stable under social stress conditions in rainbow trout (Ida Beitnes Johansen, unpublished data).

Isolation of Total RNA and DNase treatment

The samples were transferred from the -80°C freezer into liquid nitrogen, to keep them frozen until they were transferred into Lysing Matrix D tubes containing ceramic beads (MP Biomedicals) and 1ml TRIzol (Invitrogen). The tissues were homogenized twice using the homogenizer FastPrep-24 (MP Biomedicals), after which 750 μ l of homogenate was extracted and transferred to RNase free 1.5ml microcentrifuge tubes (Ambion) and kept at room temperature for 10 min to dissociate the nucleoprotein complexes. 150 μ l of chloroform (Sigma) was added to each sample and mixed manually for 15 s. After 5 min incubation at room temperature, the samples were centrifuged at 10,000 rpm for 20 min (4°C). Three separate phases emerged in the solution, 300 μ l of the top RNA phase was transferred to a new RNase-free tube, where 300 μ l of isopropanol (Arcus Produkter AS) was added. The mixture was incubated for 10 min at room temperature to denature the RNA and then at -20°C for 20 min to enhance the precipitation. The samples were then centrifuged at 10,000 rpm for 10 min (r.t.) to pellet the RNA. The pellet was washed twice with 500 μ l cold 75% ethanol (Arcus Produkter AS) and left to dry. The pellet was dissolved and stored in 30 μ l RNA storage solution (Ambion).

The extracted total RNA was treated with DNase to degrade all DNA that might remain in the samples. Turbo DNA free kit from Ambion was used. Buffer, DNase enzyme and isolated RNA were mixed and incubated at 37°C for 30 min, after which 1 μ l of DNase stop solution was added and allowed to react for 2 min. The samples were centrifuged at 10,000 rpm for 1.5 min (4°C), whereupon 30 μ l solution was transferred to new tubes and diluted with 270 μ l nuclease free water. The extracted, DNase treated RNA was stored at -80°C until cDNA synthesis. The protocol was performed according to the kit manual where a detailed description can be found.

RNA quantification and cDNA synthesis

Before cDNA synthesis, the concentration of each sample of extracted RNA was determined, using Nanodrop spectrophotometer. Absorbance was measured at 260 and 280 nm and the concentrations were calculated using Beer-Lambert's law which predicts that there will be a linear change in absorbance with change in concentration. Each sample was measured three times from which an average concentration was calculated.

The cDNA synthesis to be used for real-time PCR quantification was performed using SuperScriptIII First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The kit is optimised for synthesis of cDNA for use in real-time quantitative RT-PCR and it supports accurate quantification of RNA up to 1µg of total RNA. In the protocol we decided to use 500 ng of RNA in each reaction calculating the correct amount of RNA using concentrations obtained with Nanodrop. DEPC (Diethylpyrocarbonate) water was added to dilute the RNA to 500 ng pr reaction.

The 2X Reaction Mix, RT Enzyme Mix, RNA and DEPC water were mixed according to the protocol provided in the kit. The samples were at the following temperatures respectively; 25°C for 10 min, 50°C for 30 min and 85°C for 5 min to terminate the reaction. Samples were chilled on ice and administered RNase H, to degrade RNA in the RNA/DNA hybrid helix, with 20 min incubation at 37°C. The cDNA was diluted ten times and stored at -80°C until use in qRT-PCR. A more detailed description of the protocol can be found in the kit manual.

Real-Time PCR

The real-time PCR was carried out on a LightCycler 480 (Roche), using LightCycler 480 SYBR Green 1 Master Mix (Roche). Relative expression of a gene was calculated in comparison to a reference gene, in our case β -actin (stability of β -actin in social stress experiments had been confirmed through comparison with external standard, unpublished data by Johansen) using primer efficiency (E) and the Cp-value (formula 1). Cp-value is the point where increase in fluorescence, namely the product, is highest. The more cDNA there is at the start of the real-time reaction, the lower the Cp-value will be.

$$\text{Relative expression} = \frac{(E^{Cp})_{ref\ gene}}{(E^{Cp})_{target\ gene}} \quad (1)$$

Real-time PCR was run using the following program: Incubation for 10 min at 95°C, 42 cycles of PCR with: DNA melting 10 s at 95°C, primer annealing 10 s at 60°C and enzymatic replication 10 s at 72°C where the levels of fluorescing product were measured. Melting curves for each primer pair were recorded with a steady raise in temperature from 65-97°C lasting 15 s. Program ended with cooling of the plate at 40°C for 10 s. The genes in question and their corresponding primers are shown in table 1.

Table1. Genes and primer sequences used in the Real-Time PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
B-Actin	AGC CCT CCT TCC TCG GTA T	AGA GGT GAT CTC CTT GTG CAT C
BDNF	GAC CAA GGA TGT CGA CCT GT	GCT GTC ACC CAC TGG CTA AT
PCNA	AGC AAT GTG GAC AAG GAG GA	CGG CTA TCT TGT ACT CCA CCA
NeuroD	CCT TTA GGA GAA GTG CGG ATA	ATT GGC CCA AGT ATT CGT TT

Radioimmunoassay (RIA) for cortisol

Plasma cortisol levels were determined using a radioimmunoassay based on the assay by Pottinger and Carrick, (2001). Steroids were extracted from plasma samples by vortex mixing with ethyl acetate (Merck, 1:5 plasma:ethyl acetate) followed by centrifugation, 14,000 rpm for 2 min. An aliquot of the extract (50–200 µl) was transferred to 1.5 ml eppendorf tubes and a standard curve was constructed by adding to a series of tubes aliquots of ethyl acetate containing between 12.5 and 800 pg of inert cortisol (Sigma Aldrich). A zero sample and a nonspecific binding (blank) control were made with pure ethyl acetate. All samples, standards and controls were run in duplicate. A 50 mL aliquot of ethyl acetate with approximately 15,000 cpm of [1,2,6,7-³H] cortisol (Amersham Pharmacia Biotech; 60 Ci/mmol) was added to all the tubes and the solvent was evaporated in a dessicator coupled to a water-jet pump. A 200 ml aliquot of anti-

cortisol antibody (Donkey anti cortisol, AbD Serotec, 1:600) in assay buffer; phosphate-buffered saline (PBS tablets, Sigma) containing bovine serum albumin (Sigma, 0.1%), was added to each tube (except the blank tubes that received only assay buffer). The tubes were incubated overnight at 4°C. After incubation, racks containing the tubes were placed on ice and 100 mL of chilled, stirred, dextran-coated charcoal in PBS (1.0% activated charcoal, Sigma, 0.2% dextran, Sigma) was added to each tube. After vortex mixing, the tubes were incubated on ice for 10 min before being spun (8000 rpm at 4°C for 10 min). A 200 µl aliquot of the supernatant was added to 4.5 ml of scintillation fluid (Ultima Gold, PerkinElmer) in a scintillation vial, mixed by inversion, and counted under standard ³H conditions on a Packard Tri-Carb 1900. Cortisol concentrations in the unknown samples were calculated from the equation of a 3-parameter hyperbolic function fitted to a plot of the percentage of [³H]cortisol bound against picograms of inert cortisol (Sigmaplot 11; SPSS Science). Sensitivity (minimal detection limit) of the assay was 0.1 ng/ml.

Statistics

All statistical analysis was performed in STATISTICA for Windows (StatSoft, Inc., Tulsa, Oklahoma), while gene expression data fulfilled the criteria for parametric statistics and were compared between groups by two way ANOVA with size and treatment being the two parameters, using Tukey post-hoc test when a significant interaction was detected. Data on plasma cortisol lacked variance homogeneity (Lvene's test), and was log-transformed prior to analysis. T-test was used to compare growth rate between the two size classes small and large fish.

Results

Fish behavior

Upon removal of the wall between adjoining compartments on day eleven, clear dominance-subordinate relationships were formed which did not change during the course of the experiment. The larger fish in each pair became dominant as was expected, but in two pairs the relationship was slightly ambiguous as the smaller fish in these pairs displayed aggressive behavior, but only during feeding. These pairs were removed from the experiment. There was no difference in growth rate between large and small fish, with the two size classes growing at an average of 0.42 ± 0.08 (small fish) and 0.38 ± 0.11 % B.W. / day (large fish) during the time course of the study (t-test, $p=0.73$).

The fish interacting in pairs were filmed for 10 min after the conclusion of the fight, and for 10 min prior to feeding the following three days. Aggressive acts seen on these films were recorded. Over time, there was a marked decrease in aggressive behavior towards the subordinate fish (Figure 3.)

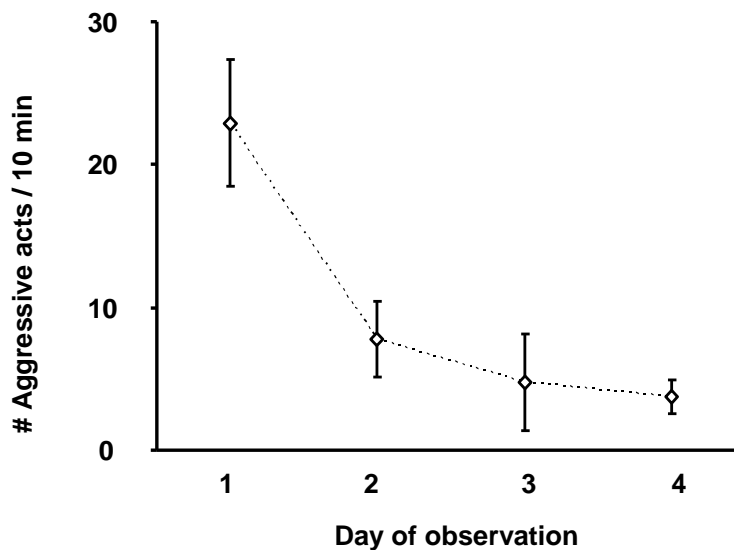


Figure 3. Overview of aggressive acts performed by the dominant fish towards the subordinate fish during 10 min following the settlement of fights for dominance (day 1) and then prior to feeding for the remainder of the study period (mean \pm S.E.M.)

Plasma cortisol

Mean and S.E.M values of plasma cortisol levels for all four experimental groups are shown in figure 2. There was no significant difference in cortisol levels between size classes (ANOVA; $F_{(1,32)} = 0.80$, $p=0.38$) or treatments (ANOVA; $F_{(1,32)} = 1.74$, $p=0.20$), but there was a trend towards an interaction between the two factors (ANOVA; $F_{(1,32)}=3.63$, $p=0.07$). Thus, although high cortisol values were seen in some subordinate fish (small interacting), this effect did not quite reach statistical significance. It should be noted, however, that the experimental protocol yielded a more subtle effect: inter-individual variation was significantly larger in small subordinate fish than in any other group (Levene's test $p=0.03$). In figure 4 original values are presented (ng cortisol/ml plasma), while the ANOVA was performed on log-transformed data, a transformation which yielded variance homogeneity between groups (Levene's test, $p=0.71$).

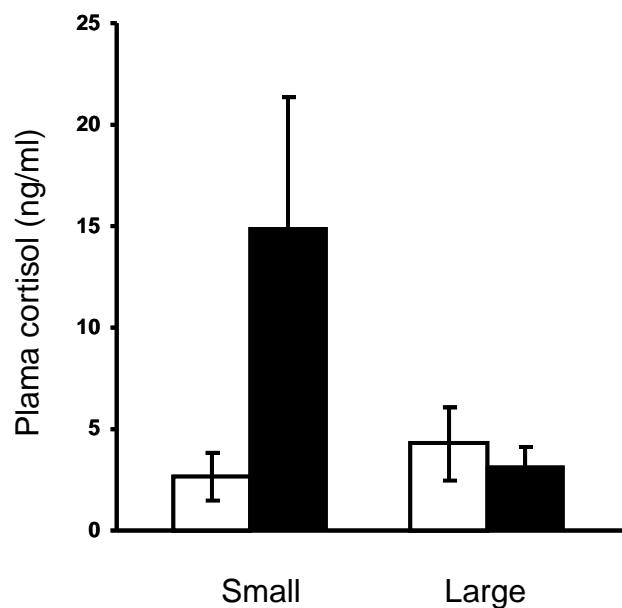


Figure 4. Plasma cortisol levels in juvenile rainbow trout. No significant effect was seen of either size class or social interaction, although there was a trend towards elevated cortisol in small interacting fish. See text for ANOVA statistics and significance level. Values are means \pm S.E.M

Gene expression

In two of the five brain parts examined, brain stem and telencephalon, there were no significant effect on expression levels of any of the three investigated genes. Therefore, only results from the remaining brain parts hypothalamus, cerebellum, and optic tectum are presented graphically. In these regions at least one gene was affected by the controlled variables: size class and/or social interaction. For reference, detailed ANOVA statistics for all genes and brain parts are given in table 2.

Expression of the cell proliferation marker PCNA is shown in figure 5. The only significant effect on this gene was a difference between size classes in the optic tectum, with small fish showing enhanced expression levels compared to large fish (Figure 5C, ANOVA: $F_{(1,37)}=12.2$, $p=0.001$). A similar pattern was seen for the neurotrophic factor BDNF: also for this gene a difference between size classes was apparent in the optic tectum (ANOVA; $F_{(1,37)}=8.94$, $p=0.005$), with a non-significant trend towards increased BDNF expression was indicated in socially interacting fish compared to isolated fish (ANOVA; $F_{(1,37)}=3.14$, $p=0.08$, c.f. figure 6C, table 2)

Regarding effects of social status alone, real-time PCR quantification indicated a significant effect on gene expression only in the hypothalamus (figure 7A). In this brain part, significant differences in the expression of the transcription factor NeuroD was found, related both to treatment (interacting *vs* isolated) (ANOVA; $F_{(1,36)} = 14.9$, $p < 0.001$), size class (ANOVA; $F_{(1,36)} = 17.4$, $p < 0.001$), and interaction between these two factors (ANOVA; $F_{(1,36)} = 23.7$, $p < 0.001$). There was a marked up-regulation in expression of NeuroD in small subordinate fish, compared to both small controls, large controls and large dominant fish (figure 7A). A similar trend towards elevated NeuroD expression in small, socially subordinate fish was seen in the optic tectum (c.f. figure 7C), but here the interaction effect did not quite reach statistical significance (ANOVA; $F_{(1,37)} = 3.86$, $p=0.06$). NeuroD also differed significantly between experimental groups in the cerebellum (Figure 7B), but here the observed pattern deviated from what was seen for NeuroD and other neurogenesis related genes in other brain parts, as it appeared that large fish showed higher expression levels than small fish, with a borderline significant effect of treatment (effect of size class: $F_{(1,36)} = 11.0$; $p = 0.002$, effect of pairing: $F_{(1,36)} = 4.07$, $p = 0.051$; interaction between factors: $F_{(1,36)} = 0.86$, $p = 0.36$).

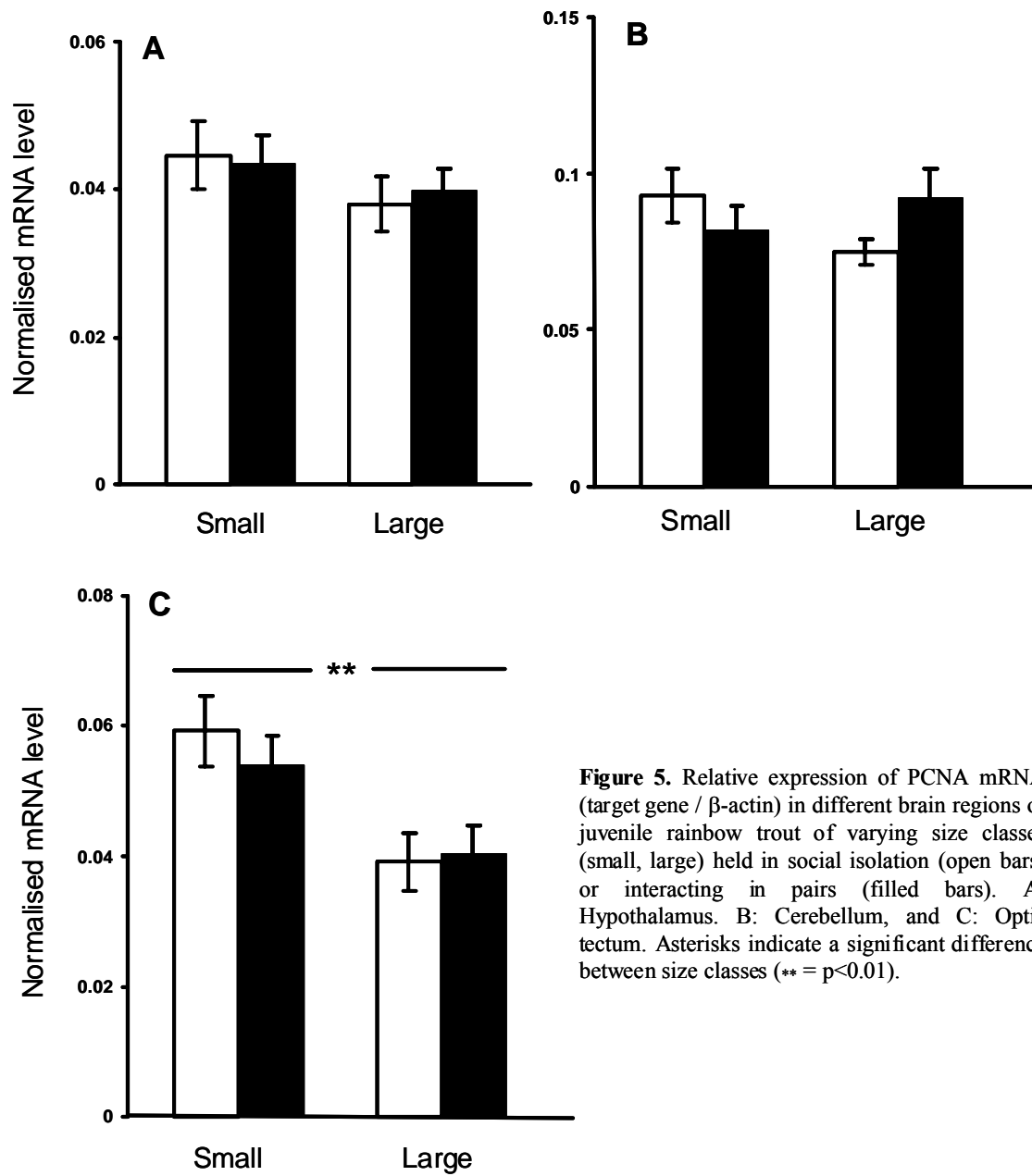


Figure 5. Relative expression of PCNA mRNA (target gene / β -actin) in different brain regions of juvenile rainbow trout of varying size classes (small, large) held in social isolation (open bars) or interacting in pairs (filled bars). A: Hypothalamus. B: Cerebellum, and C: Optic tectum. Asterisks indicate a significant difference between size classes (** = $p < 0.01$).

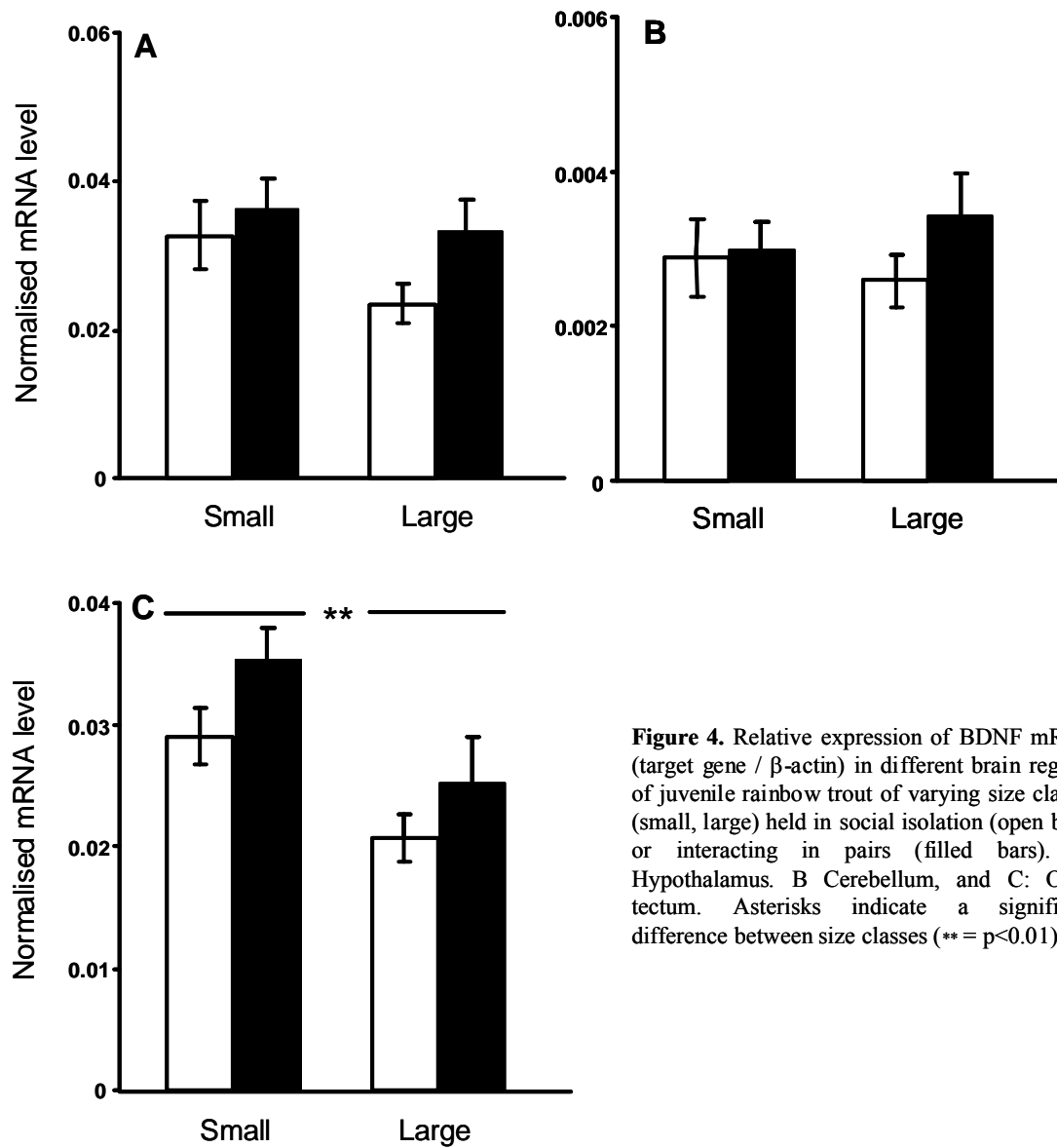


Figure 4. Relative expression of BDNF mRNA (target gene / β -actin) in different brain regions of juvenile rainbow trout of varying size classes (small, large) held in social isolation (open bars) or interacting in pairs (filled bars). A: Hypothalamus. B Cerebellum, and C: Optic tectum. Asterisks indicate a significant difference between size classes (**= $p < 0.01$).

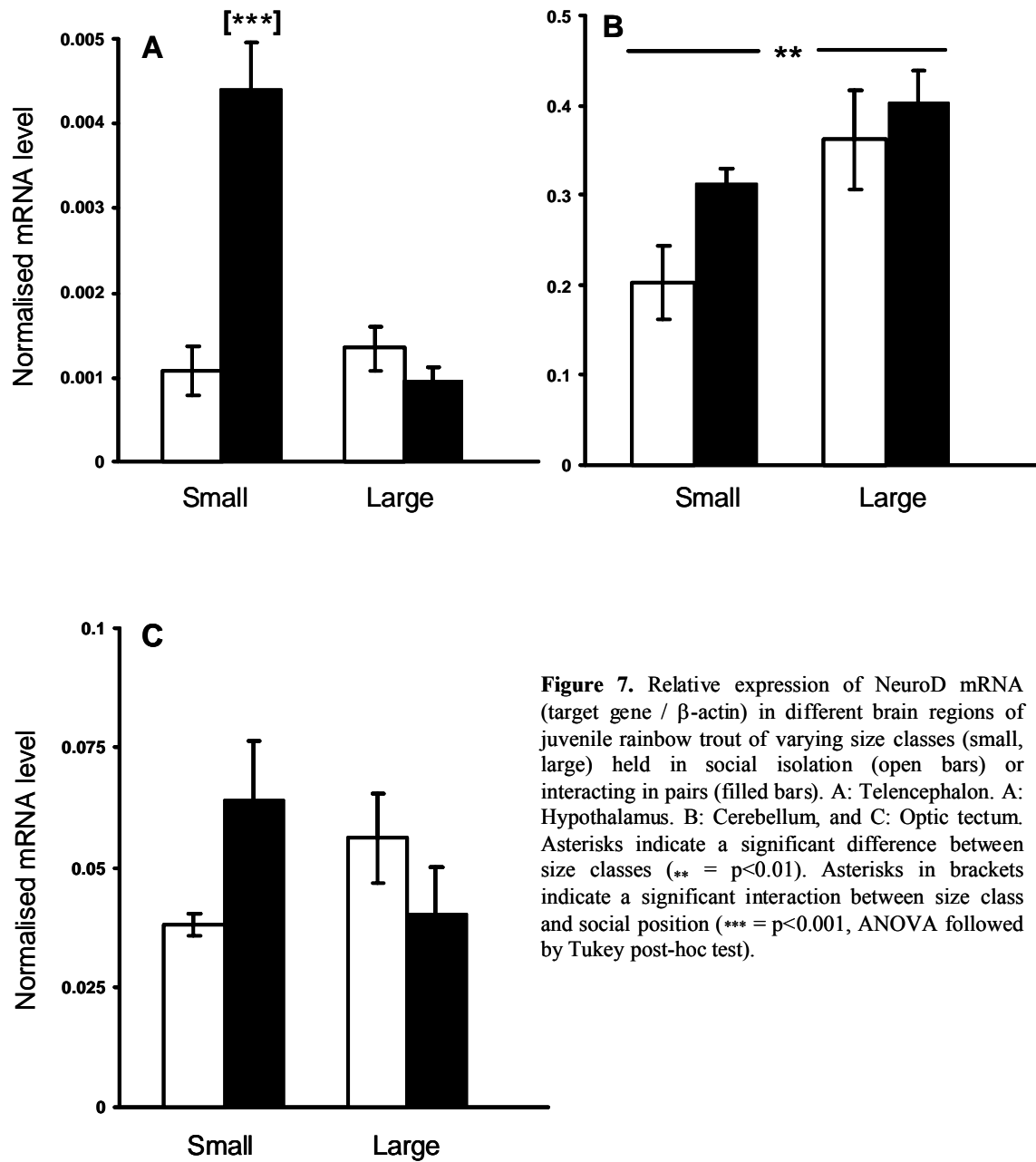


Figure 7. Relative expression of NeuroD mRNA (target gene / β -actin) in different brain regions of juvenile rainbow trout of varying size classes (small, large) held in social isolation (open bars) or interacting in pairs (filled bars). A: Telencephalon. B: Hypothalamus. C: Cerebellum, and D: Optic tectum. Asterisks indicate a significant difference between size classes (** = $p < 0.01$). Asterisks in brackets indicate a significant interaction between size class and social position (** = $p < 0.001$, ANOVA followed by Tukey post-hoc test).

Table 2. Summary of ANOVA statistics. Significant results are marked by bold font

Sample	Statistic	Size effect	Treatment effect	Interaction effect
Telencephalon	F	1.50	0.79	0.26
BDNF	P	0.23	0.38	0.61
Telencephalon	F	1.99	0.90	2.05
PCNA	P	0.17	0.35	0.16
Telencephalon	F	0.95	0.38	1.46
NRD	P	0.34	0.54	0.23
Hypothalamus	F	2.11	2.64	0.54
BDNF	P	0.15	0.11	0.47
Hypothalamus	F	1.84	0.01	0.13
PCNA	P	0.18	0.92	0.72
Hypothalamus	F	17.4	14.9	23.7
NRD	P	<0.001	<0.001	<0.001
Cerebellum	F	0.03	0.96	0.60
BDNF	P	0.86	0.33	0.44
Cerebellum	F	0.20	0.12	2.63
PCNA	P	0.66	0.73	0.11
Cerebellum	F	11.0	4.07	0.86
NRD	P	0.002	0.051	0.36
Optic tectum	F	8.94	3.14	0.086
BDNF	P	0.005	0.08	0.77
Optic tectum	F	12.2	0.15	0.50
PCNA	P	0.001	0.70	0.49
Optic tectum	F	0.06	0.24	3.86
NRD	P	0.80	0.63	0.06
Brain stem	F	0.001	0.11	0.003
BDNF	P	0.97	0.74	0.96
Brain stem	F	0.009	0.42	0.46
PCNA	P	0.92	0.52	0.50
Brain stem	F	0.34	0.39	0.59
NRD	P	0.56	0.53	0.45
Plasma	F	1.80	1.74	3.63
cortisol	P	0.38	0.20	0.07

Discussion

The methodology used in this study allowed us to obtain gene expression data from the three genes in all brain regions investigated. The study was designed to reveal differences in expression between experimental groups in relation to size class and social position. With respect to previous studies on mammals (Gould et al., 1997; Kozorovitskiy and Gould, 2004), it is perhaps somewhat surprising that differences in gene expression between experimental groups were present only in optic tectum, hypothalamus and cerebellum, and not in the telencephalon. The teleost telencephalon is presumed to contain homologues of the mammalian hippocampus (Bradford, 1995; Butler and Hodos, 1996; Rodriguez et al., 2002; Vargas et al., 2009) and have previously been shown to be affected by social stress in terms of both serotonin metabolism (5-Hydroxytryptamine, or 5-HT, a known stimulant of neurogenesis in mammals) and cell proliferation rate (Øverli et al., 1999; Sørensen et al., 2007). In the following we attempt to explain these findings in light of the time course by which psychosocial stress may arise in a dominant/subordinate relationship, and the possibly confounding factor that gene expression varies between brain areas and may in some regions with heavy expression be more related to growth and repair than to stress induced modulation of neuronal circuitry.

For instance, the difference in gene expression of PCNA between experimental groups in optic tectum was an effect of size class only. Among small fish, both interacting and isolated fish showed elevated PCNA expression in comparison to large fish. PCNA is a DNA polymerase co-factor, increasing the processivity of the polymerase. It is predominantly present in mitotic cells and can therefore be used as a marker of proliferating cells. In other words, cell proliferation would appear to be higher in small compared to large fish, regardless of treatment.

Several explanations can be envisaged for such an effect. Firstly, it is generally presumed that socially subordinate fish are subject to chronic stress (Winberg and Lepage, 1998; Winberg and Nilsson, 1993). This effect was not as predominant as expected in the current experiment, with plasma cortisol at an average of around 15 ng/ml in small socially interacting fish (c.f. figure 4). Previous studies have reported higher values (around 100 ng/ml, Øverli et al., 1999), even after one week of social interaction

(Winberg and Lepage, 1998). We observed a marked reduction in aggressive behavior over the course of the experiment, and in comparable numbers (# aggressive acts / min) the level of aggression is about half of what was reported by Winberg and Lepage (1998) over a similar time course.

The explanation nearest at hand is perhaps that, simply, less aggression equals less stress, and the effect of stress on brain structural processes in fish may follow the same inverted U-shape dose-response curve that has been observed in the mammalian forebrain (reviewed by McEwen et al., 1999). In general, it would seem that a low amount of stress or stress hormones is associated with behavioral and cognitive activation, and improves e.g. learning and memory (Joëls et al., 2006; Mateo, 2008), while chronic and severe exposure leads to the opposite result and impairs cognition.

It remains to be explained why this effect is seen not only in the apparently mildly stressed small fish kept in pairs together with large fish, but additionally in small isolated fish. It is a possibility that the effect represents a chronic trait associated simply with being the smallest individual in a group composed of fish of similar age, which was not reversed by 10 days of social isolation. Recent results show that an element of stress is also present in small individuals even in large groups of fish held at densities comparable to those seen in commercial aquaculture (Cubitt et al., 2008). These authors however reported that the chronic effect of social stress was a depletion of brain 5-HT neurotransmitter levels, which in mammals have been associated with decreased, not increased brain cell proliferation and neurogenesis (Balu and Lucki, 2009; Gould, 1999).

Another possibility is that increased PCNA expression in small isolated fish is associated with quicker growth in these individuals, as small fish are known to express compensatory growth when given increased access to food (Nikki et al., 2004). However, there was no difference between growth rates of small and large fish in the current study.

Effect of size in optic tectum is also seen for expression of BDNF, where small fish, like for PCNA, showed significantly higher BDNF expression than the large fish. BDNF is associated with a range of structural processes in the brain; generation, differentiation and survival of new neurons are stimulated by BDNF (Benraiss et al.,

2001; Rossi et al., 2006; Sairanen et al., 2005; Zigova et al., 1998). As opposed to PCNA, BDNF is not expressed by proliferating cells but by the surrounding tissue, guiding new neurons to their proper site of synaptogenesis and inducing long term survival of newly generated neurons. After reaching their final destination, at least 50% of the newly generated cells are eliminated through apoptosis, while the remaining cells can persist for the rest of the fish's life (Candal et al., 2005; Grandel et al., 2006; Zikopoulos et al., 2000; Zupanc et al., 2005a). Thus, BDNF induced long-term survival of neurons might be indicative of a higher neurogenesis rate and net growth of optic tectum in small fish, as more neurons survive and are incorporated into functional neuronal networks. Again, the fact that both PCNA and BDNF expression is higher in optic tectum of the small fish is a bit unexpected, since net brain size of teleosts increases with body length and mass (Birse et al., 1980b; Zupanc and Horschke, 1995a) and no increase in growth rate of small fish was observed in comparison to large fish. A more detailed study of the time course with which effects of social stress arise in fish is therefore called for to explain the increased expression of these genes in the small size class.

Difference in NeuroD expression in cerebellum was also an effect related merely to size, but the pattern was opposite to that seen for BDNF and PCNA in optic tectum. The expression of NeuroD was significantly higher in large fish, relative to the small ones. NeuroD is a transcription factor found in neuron determined cells, inducing cell cycle withdrawal and early phases of neuronal differentiation (Bedard and Parent, 2004; Hevner et al., 2006; Miyata et al., 1999; Pleasure et al., 2000). Mean PCNA expression of all four experimental groups in cerebellum was at least one order of magnitude higher than in the other brain regions (c.f. figure 5), indicating high overall cell proliferation in this brain region. Expression of PCNA and NeuroD does not overlap anatomically, as shown in immunostaining in zebrafish brain (Mueller and Wullmann, 2005). Their expression occur in different stages between cell proliferation and new neuron generation; PCNA is expressed during DNA synthesis in mitosis, while NeuroD is expressed post mitotically in a freshly neuron determined cells. Higher levels of NeuroD expression in cerebellum of large fish therefore suggests that a higher level of the proliferating cells are destined to become neurons. Again, without more large scale time

course studies, it cannot be determined whether the effect is due to different stress levels and social history, or related to size alone.

The hypothalamus is the only brain region where we observed difference in gene expression between experimental groups as an effect of both treatment (interaction vs. isolation), size (large vs. small) and interaction of these two factors. Small interacting fish, the ones subordinate in the social interaction, showed a marked increase in NeuroD compared to the other three groups. In other words; neurogenesis rate in socially subordinate fish increases in hypothalamus. This is in stark contrast to the generally accepted phenomenon that severe stress, including psychosocial stress, reduces neurogenesis in subordinate animals. Only two zones harboring adult neurogenesis are found in mammals; the subventricular zone (SVZ) lining the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Prickaerts et al., 2004). Thus only these two zones can be investigated for changes in neurogenesis in mammals. Studies in teleosts have mostly been concerned with stress induced changes in cell proliferation and neurogenesis in the telencephalon (Lema et al., 2005; Sørensen, 2005; Von Krogh et al., submitted), a region thought to harbor hippocampal function. Teleosts are remarkable in their ability regenerate damaged structures in the CNS, and dozens of cell proliferation zones have been identified throughout their brain. Production of new cells in the proliferation zones is at least two orders of magnitude higher than that in mammals (Zupanc, 2008; Zupanc and Horschke, 1995a). Since stress induced changes in cell proliferation and neurogenesis have only been studied in telencephalon, very little is known about these changes other parts of teleost brain. Thus, it is plausible that the other parts of the teleost brain could be affected by stress in a completely different way than telencephalon, or not at all. This notion is supported by an almost significant effect of treatment in optic tectum ($p=0.057$), the trend being a higher NeuroD expression interacting fish.

To conclude, the perhaps most striking finding of the present study was the apparent lack of changes in the expression of neurogenic and neurotrophic factors in most brain parts. Moreover, where a major change was seen in the hypothalamus, it was in the opposite direction of what could be expected from our present knowledge of the effect of

stress on the formation of new brain cells. However, this is perhaps not so surprising as there is very little information on the role of stress in neural plasticity of non-mammalian vertebrates and it is possible that fish, with their continuously growing brains, in these respects function quite differently from mammals. However, it is also clear from the present study that more studies are needed, initially involving more detailed time-course studies on the effects of stress, body size and social interactions on fish brain neurogenesis.

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