SOCIAL STATUS-DEPENDENT CHANGES IN BEHAVIOR AND NEUROGENESIS IN THE CRAYFISH *Procambarus clarkii*

by

CHA-KYONG SONG

Under the Direction of Donald H. Edwards

ABSTRACT

Crayfish (*Procambarus clarkii*) form dominance hierarchies, which are patterns of repeated fights with expected outcomes of winner and loser. Establishment of hierarchies allows dominants the first access to limited resources over subordinates, and leads to behavioral and cellular changes corresponding to the social status.

Here, the animals' responses to an unexpected unilateral touch, a non-social stimulus, were examined with respect to their social status and to their social context. Isolates oriented to the stimulus source with raised claws and elevated posture. Dominants also oriented to the stimulus both when tested alone and in the presence of a subordinate. Subordinates oriented to the stimulus while separated from their familiar dominant partner; however, they avoided it when tested while paired with the dominant. In subsequent tests first while semi-separated from the dominant and later while fully separated, the same subordinates displayed more orienting responses as the duration of post-fight separation increased. These results suggest that the lingering effects of recent social experience influence the behavior of subordinate animals.

During fights, crayfish release urine toward each other, providing critical chemosensory cues for establishing hierarchies. Throughout the lifespan, new neuronal precursors are added into clusters of olfactory local and projection interneurons (clusters 9 and 10). Here, the effect of pair-wise social experience on neurogenesis in these brain regions was examined using the proliferation marker bromodeoxyuridine. Groups of proliferating cells in clusters 9 and 10 formed distinctive comma shapes. The BrdU-positive nuclei in the head part of the comma were smaller and more circular than those in the tail part of the comma. Subordinates had fewer new neuronal precursors surviving in cluster 9 after 14 days than did dominants. Mitotic activity was not influenced by social status. The effect of social experience on neurogenesis remained when the effect of body growth rate on neurogenesis was removed. In conclusion, social domination enhances cell survival compared to social subordination. Although the function of these surviving newborn neuronal precursors is unknown they may enhance the learning ability of dominant crayfish.

INDEX WORDS: Social experience, Dominance hierarchy, Cell proliferation, Cell survival, Aggression, Olfaction, Crustacea

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CHA-KYONG SONG

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CHA-KYONG SONG

Major Professor: Donald H. Edwards
Committee: Charles D. Derby
Deborah J. Baro
Manfred Schmidt

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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Dedicated to Kim Chum-Chung

and to myself

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5-HT	5-hydroxy-tryptophan, serotonin
A	avoidance response
AL	accessory lobe
AN	antennal lobe
BrdU	5-bromo-2′-deoxyuridine, the proliferation marker
BrdU+	BrdU-labeled or BrdU-positive
cl 9	cell cluster 9
cl 10	cell cluster 10
D, Dom	dominant animal
H3P	anti-phospho-histone 3, the mitosis marker
H3P+	anti-phospho-histone 3-labeled or anti-phospho-histone 3-positive
HP	the distance between the final head position and the site of the probe's touch on the animal's side, in body lengths
I, Isol	isolated animal
LAN	lateral antennular neuropil
DCN	deutocerebral commissure neuropil
MPN	median protocerebrum neuropil
N	no response
O	orienting response
OGT	olfactory globular tract
OL	olfactory lobe
OLN	olfactory local interneuron
OPN	olfactory projection interneuron
ORN	olfactory receptor neuron
S, Sub	subordinate animal

I. Introduction

For many social animals including humans, learning to cope with emerging social conflicts becomes a crucial issue for survival since the essential resources, such as food, shelter, and mates, are limited (Barnett, 1975). Several strategies, such as competition or cooperation, have evolved in a way that not only reduces social conflicts but also favors an increase in reproductive success (Pusey and Packer, 1997). Consequently, the death of wild animals as a result of direct aggressive interactions among the same species is low (Blanchard and Blanchard, 1977). One of these social strategies, often observed when members of a small group of animals repeatedly compete for limited resources, is to form a dominance hierarchy (Pusey and Packer, 1997). A dominance hierarchy is established when some animals win contests repeatedly over the other animals by using force. Submissive behaviors of the subordinate animals appear to effectively suppress or down-regulate the aggressive behavior of the dominant animals. Thus, establishment of a dominance hierarchy in a small group of animals benefits both dominant and subordinate animals by helping them avoiding unnecessary fights that can cause serious body injuries or even the death of one animal (Pusey and Packer, 1997). The formation and maintenance of a dominance hierarchy are widely observed in a number of animals, such as primates (for a review, Sapolsky, 2005), mice (Bartolomucci et al., 2005), rats (Blanchard et al., 1993), lizards (Greenberg et al., 1984), birds (Carere et al., 2001; Poisbleau et al., 2005), pigs (Fernandez et al., 1994), fish (Fox et al., 1997; Pottinger and

Carrick, 2001), wasps (Markiewicz and O'Donnell, 2001), lobsters (Huber et al., 1997), and crayfish (Bovberg, 1953; Issa et al., 1999).

Not knowing the cellular mechanisms of dominance hierarchy formation and maintenance, a number of studies have examined cellular and behavioral changes as a result of dominance hierarchy formation and maintenance. For example, the level of brain derived neurotrophic factor (BDNF) is enhanced in the subventricular zone and in the hippocampus of dominant mice, whereas the level of nerve growth factor (NGF) is enhanced in subordinate mice (Fiore et al., 2005). Behaviorally, emerging dominant male mice exhibit greater locomotor and exploratory activity, while subordinate animals show reduced immune activity compared to dominant animals (Bartolomucci et al., 2001). Similar changes in the patterns of cellular and behavioral responses as a result of a dominance hierarchy formation also occur in crayfish (Yeh et al., 1996a, 1996b, 1997; Issa et al., 1999; Herberholz et al., 2001, 2003).

As an extension, this study further investigates social status-dependent changes occurring in the crayfish, *Procambarus clarkii*. The first chapter of this study examines social status-dependent changes in behavioral responses to unexpected touch after dominance hierarchy formation. The second chapter investigates social status-dependent changes in proliferation and survival of olfactory interneuronal precursors in the crayfish brain.

I.1. Dominance Hierarchy and Behavioral Changes in Social Animals

Although complex, a few patterns of a dominance hierarchy have been suggested. In one pattern of a dominance hierarchy, one dominant individual dominates over all other individuals in a group, without specific ranks among the subordinate individuals (Tordoff, 1954; Yeh, 1996b). This pattern of a dominance hierarchy is observed among domestic hens living together in a pen (Tordoff, 1954). They squabble by chasing and pecking each other's head and feathers until a dominance hierarchy is established (Tordoff, 1954). Once a dominance hierarchy stabilizes, dominance rank influences egg-laying, with subordinate animals showing the lowest performance of egg-laying (Cunningham et al., 1988).

In other pattern of a dominance hierarchy, a linear sequence of dominance rank is observed. In a linear dominance hierarchy, an alpha individual, the superior animal, dominates all other individuals in a group, a beta individual, the second superior animal, dominates all except the alpha, and so on down to the bottom of a hierarchy (Bovbjerg, 1953). This pattern of dominance hierarchy is observed among crayfish living together in a restricted area (Bovbjerg, 1953). Dominant crayfish display an elevated posture, move freely in their arena, and have unrestricted access to the limited resources, whereas subordinate crayfish show a low posture, avoid contact with dominant animals, and give way to dominant animals in competition over the limited resources (Hayes, 1975; Bruski and Dunham, 1987; Figler et al., 1999).

In some cases, the rank orders among the members of a group are not linear, with A dominating over B, B over C, and C over A. Dominant individuals can be defined as those that win most, but not necessarily all of the fights (Yeh, 1996b).

A number of behavioral changes are known to occur as a result of social subordination. In the subordinates of adult mice and rats, aggressive behaviors are replaced by submissive and defensive behaviors toward conspecifics (Siegfried et al., 1984; Blanchard et al., 1993, 1995). A reduction in appetitive and reproductive behaviors as a result of social subordination has also been reported in mice and tree shrews (D'Amato, 1988; Kramer et al., 1999). Subordinate rhesus monkeys voluntarily inhibit their associative learning ability when they are together with dominant rhesus monkeys (Drea and Wallen, 1999). However, in the absence of dominant monkeys, subordinate monkeys express their learned association as good as dominant monkeys (Drea and Wallen, 1999). On the other hand, when the same subordinate animals are tested among other subordinate monkeys, they perform well in association learning tasks (Drea and Wallen, 1999). In black-capped chickadees, the low-ranked subordinates and the middle-ranked subdominants are the first to send alarm signals when they hear hawk vocalizations against the risk of predation (Zanette and Ratcliffe, 1994). In American lobsters, dominant animals often display an aggressive posture, in which the animal takes an elevated stance with both claws raised high toward other subordinate animals, whereas subordinate animals display a submissive posture by staying low near the substrate with both claws lowered (Livingstone et al., 1980; Harris-Warrick and Kravitz, 1984). Among several species of animals that have developed a long-term dominant-subordinate relationship, changes in neuroendocrine and neurophysiological systems are known to

occur due to different degrees of social stress (Avis, 1974; Yeh et al., 1996b; Hofmann and Fernald, 2000). Therefore, inescapable subordination stress is frequently used to study the neuroendocrine mechanisms underlying behavioral inhibition, depression, and acquisition of fear conditioning (Koolhaas et al., 1990; Gould et al., 1997; Berton et al., 1999; Jasnow and Huhman, 2001; Fuchs and Flügge, 2002).

I.2. Dominance Hierarchy and Behavioral Changes in Crayfish

Dominance hierarchy formation in crayfish populations is characterized by differences in social and non-social behaviors. Dominant crayfish take an elevated stance, move about freely in their territory, and have first access to limited resources such as food, shelter, and mate (Bovbjerg, 1953; Hayes, 1975; Bruski and Dunham, 1987; Figler et al., 1999). On the other hand, subordinate crayfish adopt a submissive posture standing flat near the substrate, avoiding contact with dominant animals by staying at the periphery of the territory, and yielding in competitive interactions without further challenges (Bovbjerg, 1953; Hayes, 1975; Bruski and Dunham, 1987; Figler et al., 1999).

Dominance status also affects non-social behaviors. Burrowing activity for digging shelters increases in new social dominants regardless of the presence of their subordinate partner (Herberholz et al., 2003). In contrast, burrowing activity for forming shelters is inhibited in new social subordinates in the presence of dominants, but resumes in the absence of dominants (Herberholz et al., 2003). Similarly, an unexpected touch to the tailfan elicited an avoidance reaction from low-ranked or small crayfish (Bovbjerg, 1953; Nagayama et al., 1986). The avoidance response is also known as a dart response

in which the animal moved rapidly forward keeping their body flat near the substrate (Nagayama et al., 1986). However, the same stimuli elicited an immediate, oriented, and aggressive reaction from high-ranked or large crayfish (Bovbjerg, 1953; Nagayama et al., 1986).

The first chapter of this study examines the effect of social context on the crayfish's behavioral response to unexpected touch. Both social status and the social context at the time of the touch influenced the behavioral responses of a crayfish to an unexpected touch. Also, the behavioral responses of subordinate crayfish were influenced by the recent history of social experience.

I.3. The Occurrence of Adult Neurogenesis in Social Animals Including Crayfish

Evidence for production of new neurons (neurogenesis) in the brain of adult vertebrates was first presented in the early 1960s (Altman, 1962; Altman and Das, 1965), against the previously accepted view that new neurons were produced only during embryonic development and no new neurons could be produced during postembryonic development of animals. Now, adult neurogenesis in two regions of the vertebrate brain, the subventricular zone / olfactory bulb and the dentate gyrus of the hippocampus, is known to occur in birds (Goldman and Nottebohm, 1983), fish (Raymond and Easter, 1983; Byrd and Brunjes, 2001), rats (Kaplan and Hinds, 1977; Bayer, 1982; Kaplan et al., 1985; Crespo et al., 1986), mice (for reviews, Lennington et al., 2003; Prickaerts et al., 2004), monkeys (Gould et al., 1999c, 2001), and humans (Eriksson et al., 1998). In adult arthropods, life-long neurogenesis in the brain regions involved in olfaction and vision

has been reported in the mushroom bodies of several insects (Technau, 1984; Gu et al., 1999; Cayre et al, 2002), and in the brain of several crustaceans including crayfish (Harzsch and Dawirs, 1996; Schmidt, 1997; Sandeman et al., 1998; Harzsch et al., 1999; Schmidt, 2001; Song et al., 2004, 2005; Sullivan and Beltz, 2005).

These neurogenic brain regions possess life-long plasticity and are thought to mediate a number of chemical and behavioral events occurring during the lifespan of animals. Indeed, a number of literature, discussed below, report that the production and the survival of new neurons are regulated by many chemicals such as hormones and neurotransmitters, and a variety of life experiences such as stress, learning, and memory formation.

I.4. Chemical Factors Regulate Adult Neurogenesis

Many chemical factors can regulate adult neurogenesis. The regulatory effects of serotonin, hormones, and nerve growth factors on the level of adult neurogenesis will be mentioned briefly as examples.

Serotonin Serotonin is one of the well-known neurotransmitters that regulate the development of the nervous system in vertebrates as well as in invertebrates throughout their lives (Lauder, 1991; Goldberg et al., 1991; Benton et al., 1997). In adult rats, serotonin facilitates new neuronal production in the dentate gyrus of the hippocampus (Jacobs et al., 1998), and this serotonin-induced increase in new neuronal production is mediated by the activation of 5HT1A receptors (Radley and Jacobs, 2002;

Banasr et al., 2004). Moreover, chronic fluoxetine treatment, which prolongs serotonin availability within the synaptic cleft by blocking serotonin re-uptake, also increases the number of proliferating cells in the dentate gyrus of adult rats (Malberg et al., 2000; Malberg and Duman, 2003).

In the deutocerebrum of the crustacean brain, serotonin is indispensable for constructing embryonic brain structures such as the olfactory lobe (OL) and accessory lobe (AL) during early development (Benton et al., 1997; Benton and Beltz, 2001) and its regulatory roles continue into adulthood (Beltz et al., 2001). In adult lobsters and crayfish, the serotonergic dorsal giant neuron (DGN), located medial to and extensively innervating the ipsilateral OL and AL, has been suggested to be the source of serotonin regulating the level of cell proliferation and cell survival in the proliferation zone of olfactory interneuronal clusters (Sandeman and Sandeman, 1987, 1994; Sandeman et al., 1995; Beltz et al., 2001).

Endocrine Factors The effect of glucocorticoids, known as stress hormones, on adult neurogenesis has been well demonstrated in vertebrates. In adult rodents, proliferation of granule cell precursors in the dentate gyrus of the hippocampus is suppressed by high levels of circulating glucocorticoids, resulting in a reduction in hippocampal volume (Fuchs et al., 1997; Tanapat et al., 2002). In contrast, removal of adrenal steroids by adrenalectomy increases the number of proliferating cells in the dentate gyrus of the adult hippocampus (Cameron and Gould, 1994; Cameron and McKay, 1999). However, cellular mechanisms by which adrenal steroids suppress the proliferation of granule cell precursors are not fully understood. The suppressive effect

of adrenal steroids is, in part, known to be mediated by the activation of an NMDA receptor subtype in adult rats (Cameron and Gould, 1994; Cameron et al., 1995) and in adult tree shrews (Gould et al., 1997).

In invertebrates, the regulatory actions of juvenile hormone and ecdysone on neuroblast proliferation have been demonstrated in the mushroom body, the main associative center for multisensory information of the insect brain (Cayre et al., 1997a; Malaterre et al., 2003). Depletion of juvenile hormone, a hormone involved in larval molting and ovary maturation, suppresses neuroblast proliferation, whereas the injection of juvenile hormone restores the proliferative activity (Cayre et al., 1997a). The stimulatory effect of juvenile hormone is mediated by polyamine, putrescine. Administration of putrescine in juvenile hormone-depleted animals mimics the stimulatory action of juvenile hormone on neuroblast proliferation (Cayre et al., 1997a). Ecdysone, a steroid molecule involved in molting, is shown to inhibit neuroblast proliferation *in vivo*, however, without having observable inhibitory effects *in vitro* (Cayre et al., 1997b; Malaterre et al., 2003).

Correlation of peripheral neurogenesis with molt stage in the spiny lobster suggests regulatory roles for hormones in adult neurogenesis in crustaceans (Harrison et al., 2001). Although hormones related to the molt cycle and stress responses have been identified (Abramowitz et al., 1944; Keller and Sedlmeier, 1988; Chang et al., 1998), their effects on the levels of adult neurogenesis are currently unknown.

Growth Factors Many growth factors are known to regulate adult neurogenesis in rodents. For example, epidermal growth factor (EGF) and fibroblast-

growth factor 2 (FGF-2) are two key mitogens both *in vitro* and *in vivo* (for a review, Emsley et al., 2005). These two factors are used in neural precursor cultures to maintain cells in their mitotic and undifferentiated state *in vitro*, and their effectiveness as mitogens has been demonstrated *in vivo* (Reynolds and Weiss, 1992; Palmer et al., 1995; Craig et al., 1996; Kuhn et al., 1997). In addition to these two factors, insulin-like growth factor-I (IGF-I), transforming growth factor-α (TGF-α), and brain-derived neurotrophic factor (BDNF) (Craig et al., 1996; Kuhn et al., 1997; Arsenijevic and Weiss, 1998; Pencea et al., 2001) also play a role in regulating adult neurogenesis.

Vertebrate growth factor-like molecules also regulate different stages of neurogenesis in insects (Brogiolo et al., 2001; Malaterre et al., 2003). Both insulin and insulin-like growth factor-I (IGF-I) increase neuroblast proliferation in the mushroom body, and insulin-induced increase in proliferative activity is mediated by a polyamine pathway (Malaterre et al., 2003).

I.5. Life Experiences Regulate Adult Neurogenesis

The level of adult neurogenesis is also influenced by a number of life experiences, such as living in an enriched external environment, experiences of learning and memory and stressful experiences.

Environmental Influences and Sensory Inputs Enriched environments that provide a variety of sensory stimui enhance proliferation of newborn neuronal precursors in the dentate gyrus of the hippocampus in adult birds (Barnea and Nottebohm, 1994), adult mice (Kempermann et al., 1998; van Praag et al., 1999; Brown et al., 2003), and adult rats (Nilsson et al., 1999), in the olfactory bulb of adult mice (Rochefort et al., 2002), in the mushroom bodies of adult crickets (Scotto-Lomassese et al., 2000), and in the deutocerebrum of juvenile crayfish (Sandeman and Sandeman, 2000). In adult zebra finches, cell survival increases when birds are placed in a large heterosexual group compared to when they are placed alone or as male-female pairs (Lipkind et al., 2002). Additional studies have shown the importance of sensory inputs as well as functional sensory afferents in the regulation of neurogenesis levels (Hansen and Schmidt, 2001; Scotto-Lomassese et al., 2002).

In addition, the level of neurogenesis is regulated by circadian rhythms in the deutocerebrum of juvenile lobsters (Goergen et al., 2002), and by seasonal changes in the high vocal center (HVC) of adult canaries (Nottebohm et al., 1987), and in the lateral clusters of the brains of adult shore crabs (Hansen and Schmidt, 2004). Exercise, such as

running, also enhances cell proliferation and cell survival in the dentate gyrus of adult mice (van Praag et al., 1999).

Learning and M*emory* F*ormation* The finding that the majority of newborn cells in the dentate gyrus of the hippocampus differentiate into granule neurons has prompted the idea that the addition of new neurons into the hippocampal circuitry may be a mechanism for learning and memory formation (Cameron et al., 1993; Gould et al., 1999b; Leuner et al., 2003). Recent studies demonstrate that adult rats after receiving hippocampal-dependent learning tasks had more new granule neurons in the dentate gyrus, compared to those receiving hippocampal-independent learning tasks (Gould et al., 1999a; Ambrogini et al., 2000). Furthermore, hippocampal-dependent learning tasks such as place learning in a Morris water maze and trace eyeblink conditioning were able to rescue new granule cells from death (Gould et al., 1999a). On the other hand, learning tasks that activate but do not require the hippocampus such as delayed eyeblink conditioning and cue learning in Morris water maze did not alter the number of new granule neurons surviving in the dentate gyrus (Gould et al., 1999a).

In addition, when the number of newborn neurons in the adult hippocampus was reduced by the administration of a DNA methylating agent, which acts as a toxin for proliferating cells, adult rats showed impaired performance in trace eyeblink conditioning (Shors et al., 2001). As the toxin treatment ceased, not only cell proliferation but also the ability to acquire trace memory were recovered, suggesting that immature hippocampal neurons are involved and likely to be necessary for the formation of new hippocampal-dependent memories in the adult brain (Shors et al., 2001). Conversely, preservation of

formed spatial memory increases the level of cell survival in the hippocampus of adult rats (Gould E et al., 1999a). In adult birds, animals that had poor spatial memory performance also had lowered cell proliferation in the hippocampus (Pravosudov and Omanska, 2005). All these findings support the functional roles of newborn hippocampal neurons in learning and memory formation during adulthood (Drapeau et al., 2003).

Newborn neuronal precursors are also involved in learning and memory formation in the olfactory system of adult mice. Adult mice reared in an odor-enriched environment had significantly more surviving neuronal precursors in the main olfactory bulb compared to control animals reared in a standard environment, without affecting the level of hippocampal neurogenesis (Rochefort et al., 2002). Moreover, adult mice housed in an odor–enriched environment had improved olfactory memory, without affecting hippocampus-dependent spatial learning performance (Rochefort et al., 2002). Increased neurogenesis in the olfactory system of pregnant female mice may transiently enhance olfactory learning and memory formation to improve pup recognition ability during motherhood (Shingo et al., 2003).

In the olfactory system of adult insects, suppression of neuroblast proliferation by γ irradiation drastically impairs their odor learning ability (Scotto-Lomassese et al., 2003). Crayfish use olfactory cues during dominance fights (Breithaupt and Eger, 2002). Thus, it is likely that crayfish learn the olfactory cues released in urine in forming and maintaining a dominance hierarchy. The second chapter of this dissertation examines the level of neurogenesis as a result of dominance hierarchy formation and maintenance in crayfish and might help us understand the learning ability of an animal with respect to social status.

Stressful Experiences Chronic stress, induced by restraint, significantly reduces the number of new neurons in the dentate gyrus of the hippocampus of adult rat (Pham et al., 2003) and in olfactory regions of the brain of juvenile crayfish (Sandeman and Sandeman, 2000). After the experience of inescapable foot-shock stress, adult rats had significantly lower levels of cell proliferation in the dentate gyrus, while the experience of escapable foot-shock stress had no suppressive effect (Malberg and Duman, 2003). Subordination stress decreases the number of proliferating cells in the dentate gyrus of adult tree shrews (Fuchs et al., 1995; Gould et al., 1997), adult marmosets (Gould et al., 1998), adult male rats (Kozorovitskiy and Gould, 2004), and adult birds (Pravosudov and Omanska, 2005). Subordination stress also lowers the level of cell proliferation, but not cell survival, in juvenile Australian crayfish *Cherax destructor* living together with their dominant opponents in groups of three for two weeks (Pelz, 2001).

I.6. Social Status and Neurogenesis in Crayfish

The experience of a dominant-subordinate relationship, is not only stressful enough to induce corresponding behavioral changes but also represents learning and memory formation about the opponent. A dominant-subordinate relationship is formed when groups of socially naïve crayfish meet (Bovbjerg, 1953; Lowe, 1956). During dominance fights, crayfish release urine toward each other, which is directed to the olfactory organs of the opponent (Breithaupt and Eger, 2002), and thus olfactory learning and memory formation is likely to contribute to the formation and maintenance of a

dominant-subordinate relationship. In brain regions involved in olfaction, new neurons are continuously produced throughout the lifespan of crayfish (Sandeman et al., 1992). Thus, the focus of the second chapter of this dissertation is to examine whether the level of cell proliferation and cell survival changes after the experience of a pair-wise dominant-subordinate relationship in crayfish.

Chapter 1. Social Status and Behavior:

Changes in Behavioral Response to Unexpected Touch

CHAPTER 1

Social Status and Behavior:

CONTENTS

I. INTRODUCTION

Dominance hierarchies in crayfish populations, as in other social animals, are characterized by differences in individual behavior that span the range from the aggressive displays of the most dominant to the submissive and avoidance responses of the most subordinate. Dominant crayfish often display an elevated stance, move about freely, and compete aggressively for available resources, whereas subordinate crayfish adopt a more flat posture, move to avoid contact with dominant animals, and give way to dominants in competitive interactions (Hayes, 1975; Livingstone et al., 1980; Bruski and Dunham, 1987; Krasne et al., 1997; Figler et al., 1999).

Dominance status affects non-social behaviors as well as social behaviors. Herberholz et al. found that burrow digging increased among new social dominants and was inhibited in new social subordinates (Herberholz et al., 2003). Similarly, an unexpected touch of the tailfan elicited an avoidance reaction from low-ranked or small crayfish and an immediate, oriented, and aggressive reaction from high-ranked or large crayfish (Bovbjerg, 1953; Nagayama et al., 1986). Under the threat of unexpected attack, dominant and subordinate crayfish rely on different types of escape circuitries that produce escape response, the tailflip (Krasne et al., 1997).

To study the effect of social context on the crayfish's response to unexpected touch more completely, I examined the behavioral responses of socially inexperienced and experienced crayfish in three different social conditions: while socially isolated for a

prolonged period, while briefly isolated from a dominant or subordinate partner, and while in the presence of a dominant or subordinate partner. I found that the behavioral responses of a crayfish to an unexpected touch depended on both its social status and on the social context at the time of the touch. Part of this study was previously presented in abstract form (Song et al., 2000).

II. MATERIALS AND METHODS

II.1. Animal Preparation

Adult crayfish (*Procambarus clarkii*, Girard), of wet body weight 4–8g, were used in this study. Animals were purchased from a local supplier (Atchafalaya Biological Supply, Raceland, LA, USA) and divided into groups of 8-12 in communal tanks (20-gallon) until use. Pairs of same-sex animals, mismatched by wet body weight (>5%), were transferred to 5–gallon tanks (20 cm x 25 cm x 40 cm in W x H x L). An opaque plastic divider placed halfway along the long axis of the aquarium prevented the animals from seeing or touching each other but did not isolate them chemically. The bottom of each tank was covered with gravel and the tanks were filled with continuously aerated de-chlorinated tap water. Animals were each fed with five shrimp pellets three times a week and maintained on a 12hr:12hr light/dark cycle.

II.2. Preparation of Socially Inexperienced and Experienced Animals

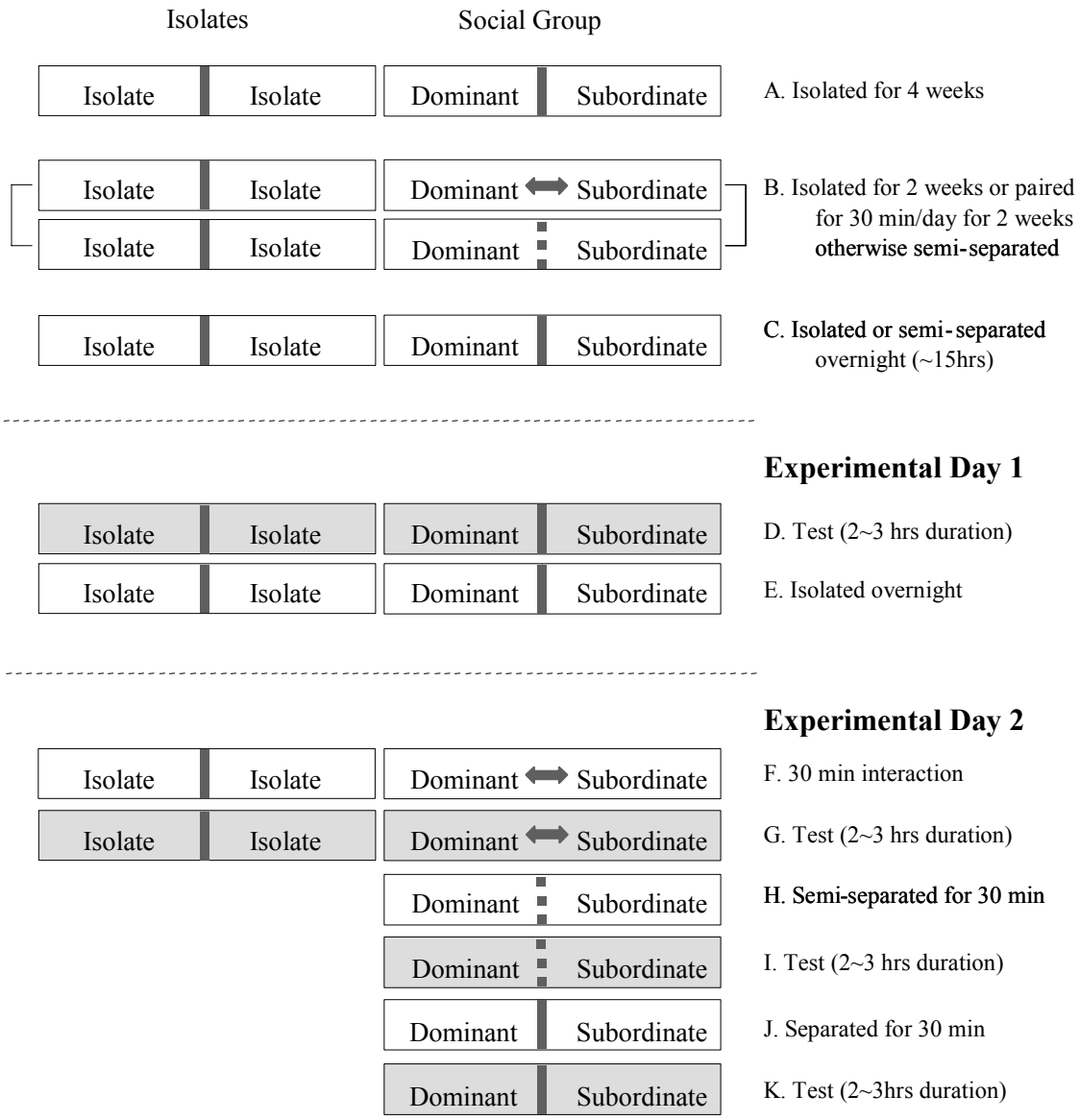
Twenty-eight animals were isolated in their divided aquaria for 4 weeks without disturbance to eliminate the influences of previous social history (Yeh et al., 1996a, 1996b, 1997) (Fig. 1A). Eight animals ('Isolates' in Fig. 1) remained isolated thereafter with their opaque dividers intact (Fig. 1B), while members of ten pairs ('Social Group' in Fig. 1) were then allowed to interact and form a dominance hierarchy by removing their dividers for 30 min / day for two weeks. One of the isolated animals died during molting and two subordinate animals died or were severely injured during pairing, leaving seven isolates and eight pairs for analysis. The eight pairs were monitored continuously during their daily 30 min periods of interaction. Six pairs retained the same dominant-subordinate relationship throughout the period of pairing, however, two pairs experienced status reversal. Status reversals did not occur during the period of experiments. Social status was determined during each 30 min period of interaction by scoring the numbers of attacks, approaches, retreats, and tailflips (escapes) of both members of a pair. The animal that made a higher dominance score (attack, +2; approach, +1; retreat, -1; tailflip, -2) was identified as the dominant, and its opponent as the subordinate. Dominance score (D) was calculated for all pairs during the pairing period according to equation 1:

$$D = \frac{100 \times (2 \times \#Attacks + \#Approaches - \#Retreats - 2 \times \#Escapes)}{2 \times \#Attacks + \#Approaches + \#Retreats + 2 \times \#Escapes} \quad (1)$$

The animals were "semi-separated" between daily interaction sessions by an open plastic square lattice (the plastic strips were 9 mm thick, 20 cm deep, and the square openings were 15 x 15 mm wide) (Fig. 1C). This open divider allowed the paired

animals to see, touch, and smell one another, but prevented vigorous fighting. For the eight pairs in Social Group, an opaque divider replaced the open divider at the end of the two weeks of pairing to establish isolated conditions for subsequent experiments.

Fig. 1 A time chart of animal preparation and experiments. Pairs of animals share aquaria (rectangular boxes), where they can be isolated from each other by an opaque barrier (gray vertical line), kept apart by an open divider that allowed pairs to smell, see, and touch each other but not to fight (dashed gray line), or interact freely (gray double-headed arrow). The sequence of treatments is shown vertically, in rows labeled from **A** to **K**. Tests are indicated by light coloring of the rectangles. Animals were divided into two groups, Isolates and social pairs. **A**. All animals were separated from an aquarium partner by a closed divider (i.e., isolated) for four weeks. **B**. (two rows) Isolates were separated for two more weeks and thereafter through the tests. Social pairs were allowed to interact freely and form dominance relationships for 30 min / day; at other times they were kept apart by an open divider. Dominance status was determined during each 30 min of interaction by scoring the numbers of aggressive and submissive behaviors performed by each member of a pair (See equation 1 in Materials and methods, and Fig. 3). **C**. At the end of the two weeks, the social pairs were re-isolated overnight "(~ 15 hours) to prevent further agonistic interactions. **D**. On experimental day 1, Isolates and the socially paired animals were tested while separated. **E**. Isolates and socially paired animals remained separated overnight. **F**. On Day 2, Isolates remained separated while social pairs were allowed to interact freely with their partners for 30 min. **G**. All animals were tested, Isolates while separated and social pairs while in the presence of their partner. **H**. Social pairs were kept apart by an open divider for 30 min, and then (**I**) tested over a 2-3 hour period. **J**. Social pairs were then re-isolated by a closed divider for 30 min, and then (**K**) tested again over 2~3 hours while isolated.


Isolates
Social Group
Isolate
Isolate
Dominant
Subordinate
A. Isolated for 4 weeks
B. Isolated for 2 weeks or paired for 30 min/day for 2 weeks otherwise semi-separated
C. Isolated or semi-separated overnight (~15hrs)
Experimental Day 1
D. Test (2~3 hrs duration)
E. Isolated overnight
Experimental Day 2
F. 30 min interaction
G. Test (2~3 hrs duration)
H. Semi-separated for 30 min
I. Test (2~3 hrs duration)
J. Separated for 30 min
K. Test (2~3hrs duration)

II.3. Experiments on Day 1

On the next morning, all animals were tested over a three-hour period (Day 1, Fig. 1D). Isolates and paired animals remained isolated by a closed divider during testing. A minimum of five manual touches were delivered in alternation to each side of the first abdominal segment with a fine brush by an experimenter working under dim red light and ignorant of the social status or experience of the animal undergoing tests. Each touch stimulus was delivered to minimize disturbances of the water, which would alert the animal to the approach of the brush, so that the animal was unexpectedly touched. Responses were recorded on videotape (Panasonic, WV-BP500, 30 frames / second) for later analysis. The interval between touches was ten minutes. Tests were performed when the animals were stationary and not interacting. Following the experiment, the opaque dividers were replaced to separate the dominant and subordinate for approximately 15 hours overnight (Fig. 1E).

II.4. Experiments on Day 2

The opaque dividers between the pairs were removed to allow the dominant and subordinate animals to interact freely (Fig. 1F). After 30 min of interaction, these animals were tested for 2-3 hours while together, whereas the Isolates were tested while remaining isolated (Fig. 1G). After the initial tests, open dividers were placed between the dominant and subordinate animals for 30 min to minimize physical contact between them while permitting chemical signaling (Fig. 1H). The animals were then tested with the open dividers still in place (Fig. 1I). After these tests, the open dividers were replaced with opaque dividers to re-isolate the paired animals for 30 min. The animals

were then tested a final time while isolated (Fig. 1J, 1K). Each set of tests took 2-3 hours to perform.

II.5. Quantification of Behavioral Responses

Video images containing the positions of an animal before and after each manual touch (stimulus) were captured using Scion image (Scion Image, NIH) (Fig. 2). Behavioral responses were examined and categorized into 'Orienting', 'Avoidance', and 'No Response' groups. Orienting responses were movements immediately following the touch that reoriented the animal to face the stimulus source (Fig. 2A). The category includes three behaviors: a turn towards the stimulus source, a brief walk backward while turning to face the stimulus source, or a short backward jump with a brief abdominal flexion to face the stimulus source. Avoidance responses were movements away from the stimulus source. These included six behavioral responses: the animal rapidly walked forward away from the site of the touch (Fig. 2B), it walked backward away, it walked sideways away, it rotated the body axis away from the stimulus source without walking, or it tailflipped away. The No Response behavior included all those in which the animal remained in the same position. Movements or responses that were initiated two seconds or more after the manual touch were not considered.

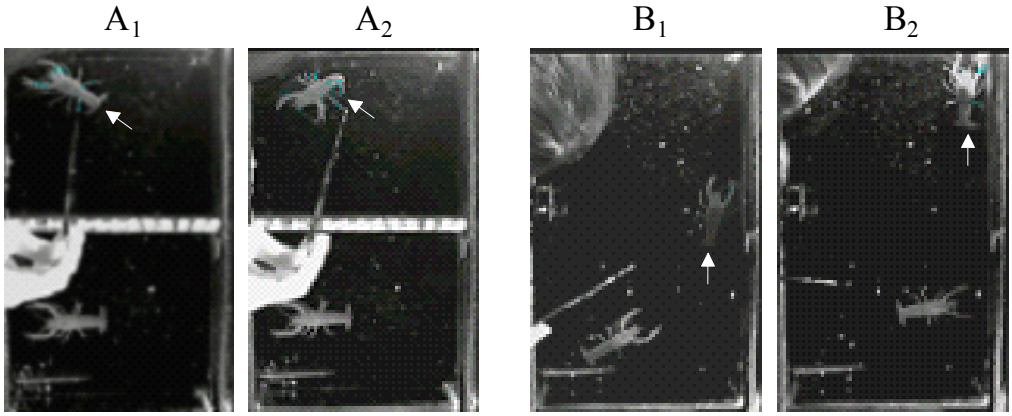
A quantitative description of the movements was obtained by marking the position of the stimulus probe at the time of the manual touch (P), and the positions of the animal's head (H) and the center of body mass (C) after the manual touch (Fig. 2C-2D). Using these marks, the head to probe distance value (HP distance value = HP distance / body length) and the angle gamma (γ; the angle between the body axis and the HP vector,

see Fig. 2C-2D) were obtained. The HP distance decreased when an animal moved toward the stimulus probe (Fig. 2C), whereas it increased when an animal moved away from the stimulus probe (Fig. 2D). The angle γ tended to decrease either when the animal turned to orient towards the probe (Fig. $2C_1$) or moved a body length or more backward to avoid the probe (Fig. $2D_1$). The angle γ was large when an animal did not move, turned toward the stimulus probe and moved forward, beyond the probe position (Fig. $2C_2$), or moved forward (Fig. $2D_2$).

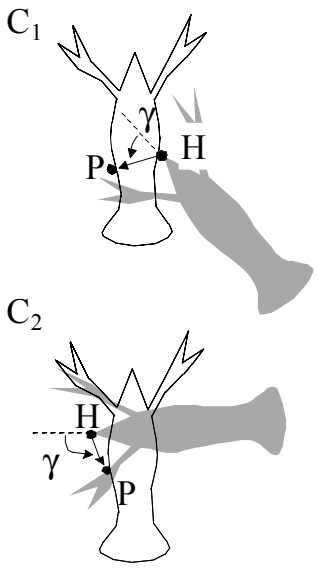
II.6. Analysis of Behavioral Responses to Touch

The behavioral response patterns of the isolate, dominant, and subordinate animals were compared using Kruskal-Wallis test (nonparametric one-way ANOVA). When an overall significant difference was found in the behavioral response patterns of the three social types of animals, Mann Whitney test (nonparametric two-tailed t-test) was used to identify which type of animals differed. Friedman test was used to examine the overall difference among the response patterns of the dominant and subordinate animals in the four consecutive social conditions; separated, paired, semi-separated, and separated. Wilcoxon signed rank test was used for pair-wise comparison only when there was an overall significant difference throughout the four different behavioral response patterns.

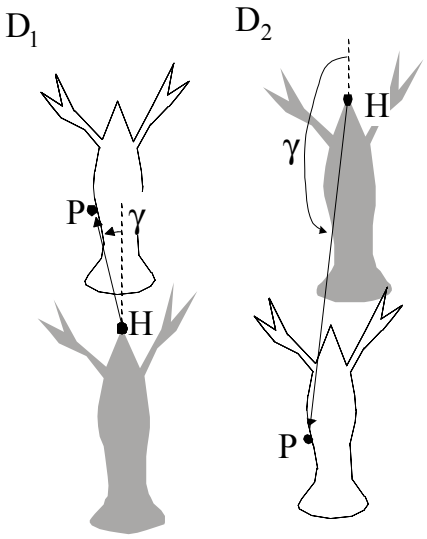
Fig. 2 Experimental tests and measures. **A**. Response of a subordinate crayfish (see arrows) separated from its dominant partner by an opaque divider. $\mathbf{A_1}$. Before the stimulus touch. $\mathbf{A_2}$. After the touch; the subordinate animal has turned to face the source of the touch. **B**. Response of a subordinate (see arrows) in the presence of its dominant partner. $\mathbf{B_1}$. Before the stimulus touch. $\mathbf{B_2}$. After the touch the same subordinate animal moved forward away from the touch site. After the subordinate moved forward, the other animal, the dominant, slowly approached to the location where the subordinate animal was touched **C** and **D**. Diagrams describing measurements of the HP distance and the angle γ values in orienting and avoidance responses. Open crayfish outline, the position of the animal before stimulus; gray crayfish, the position of an animal after stimulus. The HP distance is the distance from the head (H) to the point of probe contact (P). γ is the angle between the body axis and the HP line segment. **C**. Orienting responses. $\mathbf{C_1}$. Both γ and HP are small when an animal pivots backward to face the probe. $\mathbf{C_2}$. γ is large and HP is small when an animal turns and advances past the probe. **D**. Avoidance responses. $\mathbf{D_1}$. γ becomes small and HP large when an animal walks backwards away from the probe. $\mathbf{D_2}$. γ and HP both become large when an animal walks forward away from the probe.



C. Orienting responses



D. Avoidance responses

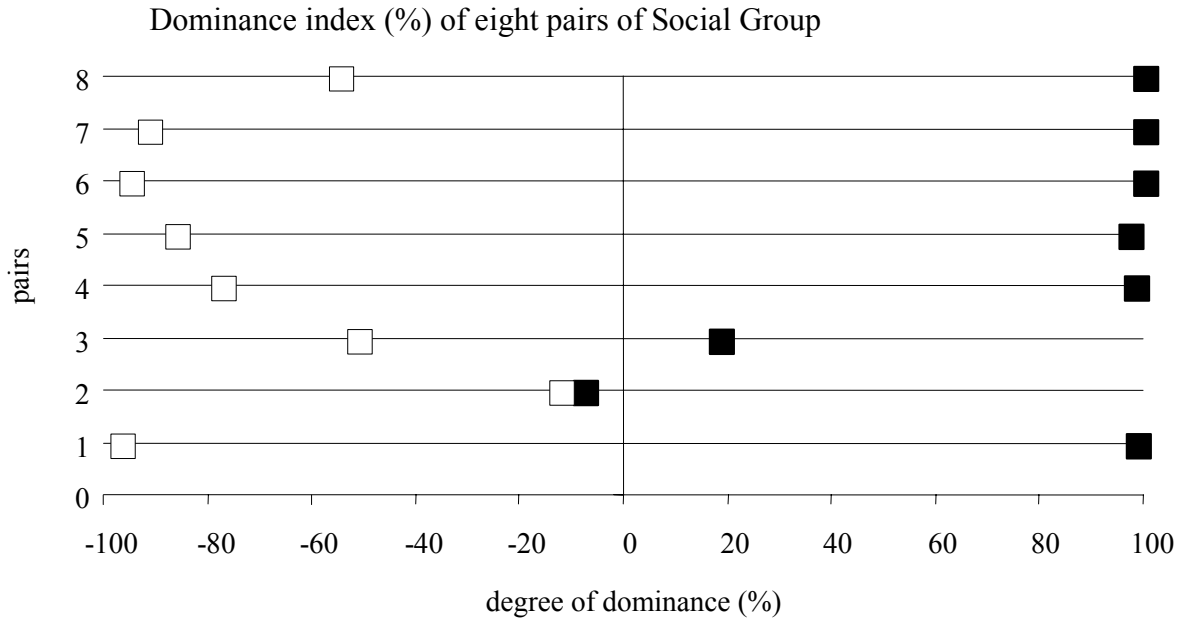


III. RESULTS

III.1. Social Hierarchy Formation Induced by Daily Pairing

Eight pairs of animals that were allowed to interact for 30 min / day for two weeks formed dominant / subordinate relationships as indicated by their scores in a dominance index (attack, +2; approach, +1; retreat, -1; tailflip, -2). Six out of the eight pairs of animals in Social Group formed stable dominant / subordinate relationships (Fig. 3), while status reversals occurred in two pairs (pairs 2 and 3 in Fig. 3), after one animal in each pair molted. No status reversals took place during the experiments.

Fig. 3 Dominance index of eight pairs of crayfish recorded during the pairing period (Fig. 1B). All aggressive and submissive behaviors that were recorded during the daily 30 min pairing were scored (attack, +2; approach, +1; retreat, -1; tailflip, -2) and presented as a percentage to show the degree of dominance between each dominant/subordinate pair (see equation 1, Materials and methods). The degree of dominance was higher in all dominant animals (filled squares) than in subordinate animals (open squares). The dominant animals in pairs 2 and 3 were dominant for shorter periods (2 days and 8 days, respectively, at the end of 14 days) because of status reversals.


Dominance index (%) of eight pairs of Social Group
pairs
8
7
6
5
4
3
2
1
0
-100
-80
-60
-40
-20
0
20
40
60
80
100
degree of dominance (%)

III.2. Initial Responses to Unexpected Touch

Isolates were tested while isolated; members of pairs in Social Group were tested when separated from their partners by an opaque divider (Fig. 1D). Unexpected lateral touch stimuli were delivered to animals in both groups under dim red light so that the animals could not see the approach of the brush. The response patterns of the Isolates and dominant and subordinate members of Social Group were similar (Fig. 4A; Kruskal-Wallis test, $p = 0.4570$ for Orienting Responses). The predominant response was to orient towards the stimulus: average frequencies fell between 68% and 80% for the three social classes (Isolates, dominants and subordinates of Social Group; Fig. 4A). The frequencies of Avoidance and No Responses were approximately equal in each class, and no statistical difference was found between the three classes' response patterns (Fig. 4A; Kruskal-Wallis test, $p = 0.7133$ for Avoidance Responses, $p = 0.6163$ for No Responses).

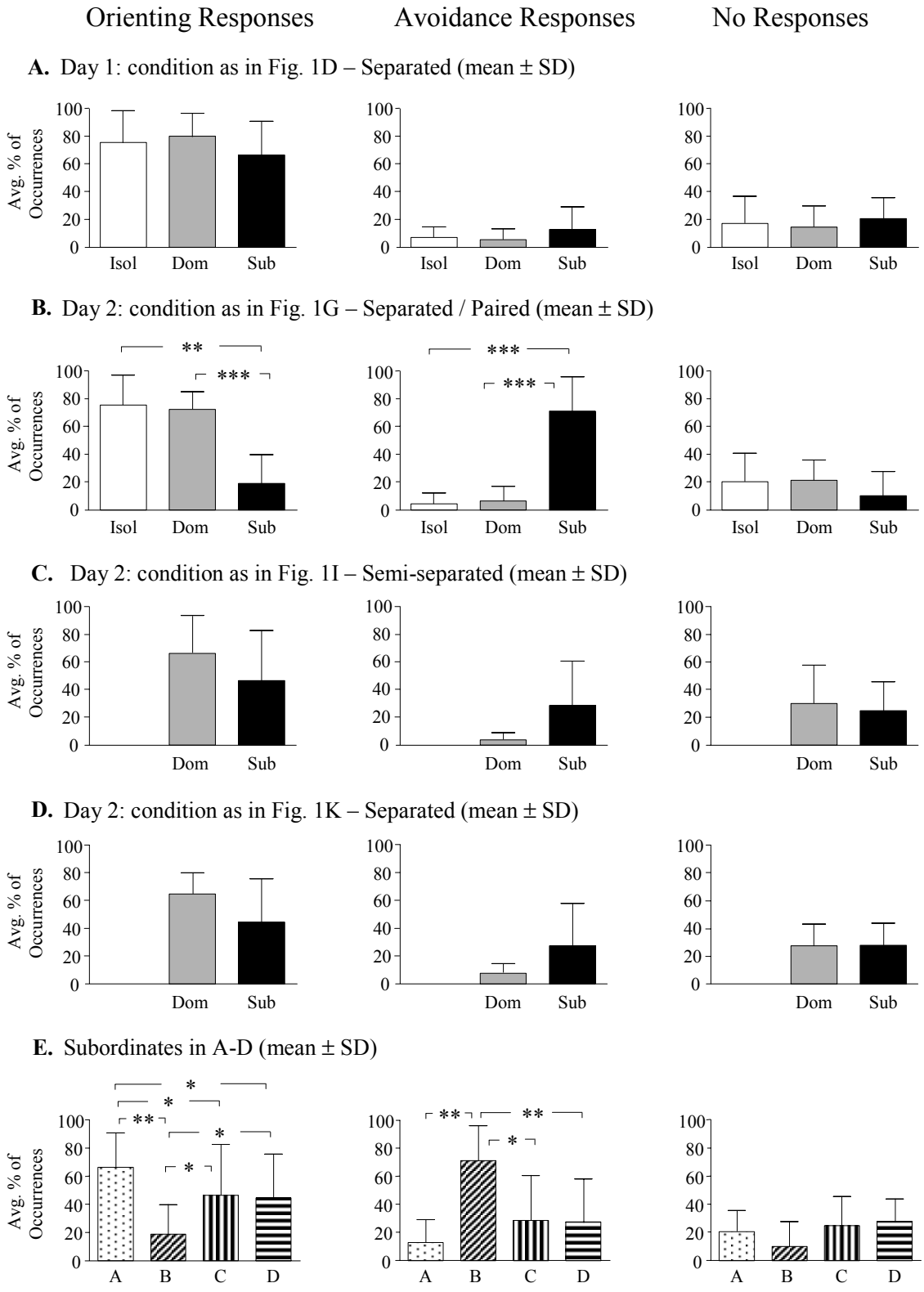
III.3. Tests While Paired, Day 2

The Isolate animals and the animal pairs of Social Group spent the next 24 hours still separated (Fig. 1E). Isolate animals remained isolated, while animal pairs in Social Group were allowed to interact freely for 30 min under dim red light (Fig. 1F), after which they were tested again (Fig. 1G). Each touch stimulus was delivered while the dominant and the subordinate animals were not moving and not interacting. The average frequencies of the Orienting Responses of the Isolates, dominants and subordinates of Social Group significantly differed (Fig. 4B; Kruskal-Wallis test, $p = 0.0008$). The

Isolates and the dominants (who were free to interact with the subordinate partners) displayed the Orienting Responses in similar frequencies to each other (Fig. 4B; Mann Whitney test, $p = 0.6126$) and to those of the previous day (Fig. 4A). More than 70% of their responses were to orient towards the stimulus; most of the remaining stimuli evoked No Response (Fig. 4B). In contrast, the subordinate animals behaved very differently from both dominants and Isolates, displaying Avoidance Responses to more than 76% of the stimuli, and Orienting Responses to only 19% (Fig. 4B). The average frequencies of the Orienting Responses displayed by the subordinates were significantly lower than those of the Isolates (Mann Whitney test, $p = 0.0012$) and dominants (Mann Whitney test, $p = 0.0002$) (Fig. 4B). Conversely, the average frequencies of the Avoidance Responses of the Isolates, dominants and subordinates of Social Group significantly differed (Kruskal-Wallis test, $p = 0.0003$), because the subordinates displayed the Avoidance Responses significantly more than the Isolates (Mann Whitney test, $p = 0.0003$) and dominants (Mann Whitney test, $p = 0.0002$). The behavioral responses of the subordinates were also different from those the day before, when the subordinates were separated from their dominant partners (Fig. 4E; Friedman test, $p = 0.0004$ for an overall significant difference; Wilcoxon test, $p = 0.0078$ for pair-wise comparison). This difference indicates that the behavioral responses of the subordinate animals were significantly affected by the presence of the dominant animals.

Fig. 4 Sequential comparison of the behavioral responses to an unexpected manual touch in isolate, dominant, and subordinate crayfish. **A**. Average response frequencies (mean±SD) of Orienting (O), Avoidance (A), and No Response (N) behavioral responses of isolates, dominants and subordinates on day 1 while separated from partners by an opaque barrier. The three behavioral responses of the isolates and social pairs did not differ significantly (Kruskal-Wallis test, $p = 0.4570$ for Orienting Responses, $p = 0.7133$ for Avoidance Responses, $p = 0.6163$ for No Responses). **B**. Average response frequencies (mean±SD) of the same animals on day 2 when the dominant and subordinate crayfish were paired with their partners. The orienting and the avoidance responses of dominants and isolates were significantly different from those of subordinates (for Orienting Responses, overall difference, $p = 0.0008$; Isolates vs subordinates, $p = 0.0012$; dominants vs subordinates, $p = 0.0002$; for Avoidance Responses, overall difference, $p = 0.0003$; Isolates vs subordinates, $p = 0.0003$; dominants vs subordinates, $p = 0.0002$), but were not different from each other (for Orienting Responses, $p = 0.6126$; for Avoidance Responses, $p = 0.7789$). The No Responses were not different in Isolates, dominants, and subordinates (overall difference, $p = 0.2069$). **C**. Behavioral responses later on Day 2 when the dominants and subordinates were tested while separated by an open divider. The dominants' responses were not different from those of the subordinate (for Orienting Responses, $p = 0.2786$; for Avoidance Responses, $p = 0.1605$; for No Responses, $p = 0.7984$). **D**. Dominant and subordinate responses after re-isolation are not significantly different (for Orienting Responses, $p = 0.2345$; for Avoidance Responses, $p = 0.3282$; for No Responses, $p = 0.9591$). **E**. The behavioral responses of the subordinates in the four consecutive social

conditions (shown in A-D) significantly changed after pairing experience (Friedman test, $p = 0.0004$ for both Orienting and Avoidance Responses). The subordinate animals when isolated on day 1 displayed the Orienting Responses significantly more often than when paired on day 2 (Wilcoxon test, $p = 0.0078$), when semi-separated on day 2 ($p = 0.0313$), and when isolated on day 2 ($p = 0.0313$). The subordinate animals when paired on day 2 displayed the Orienting Responses significantly less often than when semi-separated on day 2 ($p = 0.0156$) and when isolated on day 2 ($p = 0.0156$). The average frequency of the orienting response when semi-separated on day 2 did not differ from those when isolated on day 2 ($p = 0.5625$). The subordinate animals when paired on day 2 displayed the Avoidance responses significantly more often than when isolated on day 1 ($p = 0.0078$), when separated by the open divider on day 2 ($p = 0.0156$), and when isolated on day 2 ($p = 0.0078$). The average frequency of No Responses did not change in the four social conditions examined (overall difference, $p = 0.0660$). * stands for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$


Orienting Responses
Avoidance Responses
No Responses
A. Day 1: condition as in Fig. 1D – Separated (mean ± SD)
B. Day 2: condition as in Fig. 1G – Separated / Paired (mean ± SD)
C. Day 2: condition as in Fig. 1I – Semi-separated (mean ± SD)
D. Day 2: condition as in Fig. 1K – Separated (mean ± SD)
E. Subordinates in A-D (mean ± SD)
Avg. % of Occurrences
Isol
Dom
Sub
A
B
C
D

III.4. Test on Social Pairs Separated by an Open Divider, Day 2

To determine whether the change in the subordinate animals' responses depended on being able to interact freely with the dominants, the animals were tested again after being separated for 30 min by an open divider, again under dim red light. During this period, the subordinate animals usually stayed away from the open divider, whereas the dominants would often approach it. Both animals, especially the subordinates, often moved along the surrounding walls, touching them with their claws. When the subordinates touched the holes of the open divider, the dominants often tried to attack them, but the plastic mesh prevented serious physical interactions from occurring. The responses of the dominants were as before, with nearly two-thirds being the Orienting Responses (Fig. 4C). However, the subordinate animals displayed the Orienting and the Avoidance Responses at similar frequencies (Fig. 4C), a pattern that newly emerged in subordinates after pairing experience. Moreover, the frequencies of the Orienting Responses of the subordinate animals in a semi-separated condition was significantly different from each of the response frequencies recorded earlier (Fig. 4E), first when the subordinates were isolated on day 1 (Wilcoxon test, $p = 0.0313$), and then when they were paired on day 2 (Wilcoxon test, $p = 0.0156$). Similarly, the Avoidance Responses of the subordinate animals in a semi-separated condition were significantly reduced compared to those recorded earlier when they were paired on day 2 (Wilcoxon test, $p = 0.0156$).

III.5. Tests on Re-isolated Social Pairs, Day 2

To determine whether the subordinate animals' change in behavior depended on the near presence of the dominant as communicated through the open divider, the animals were tested again when they were completely separated by an opaque divider. The behavior patterns of both the subordinate and dominant animals (Fig. 4D) were little changed from when the animals were separated by the open divider (Fig. 4C). The behavioral responses of the subordinates were neither significantly different from those of dominants (Fig. 4D; Mann Whitney test, $p = 0.2345$ for Orienting Responses, $p = 0.3282$ for Avoidance Responses), nor different from those recorded earlier when the subordinates were in a semi-separated condition (Fig. 4E; Wilcoxon test, $p = 0.5625$ for Orienting Responses, $p = 0.8125$ for Avoidance Responses). However, the subordinates when isolated on day 2 (Fig. 4D) displayed the Orienting Responses less frequently compared to when they were isolated on day 1 (Fig. 4E; Wilcoxon test, $p = 0.0313$), but more frequently compared to when they were paired on day 2 (Fig. 4E; Wilcoxon test, $p = 0.0156$). These results indicate that both the presence of the dominant animal and the lingering effects of their social interactions during the test period changed the behavioral response of the subordinate animals to unexpected touch.

There was no difference in the behavioral response of the dominant animals in the four different agonistic conditions tested (Friedman test, $p = 0.0979$ for Orienting Responses, $p = 0.09042$ for Avoidance Responses, $p = 0.2992$ for No Responses), indicating that the behavioral responses of the dominant animals were not dependent on the presence of their subordinate partners. Thus, it is either the opportunity to interact

with the dominant or the interactions themselves that led to a shift in the response patterns of the subordinate animals from Orienting to Avoidance.

III.6. Quantification of the Behavioral Responses to Manual Touch

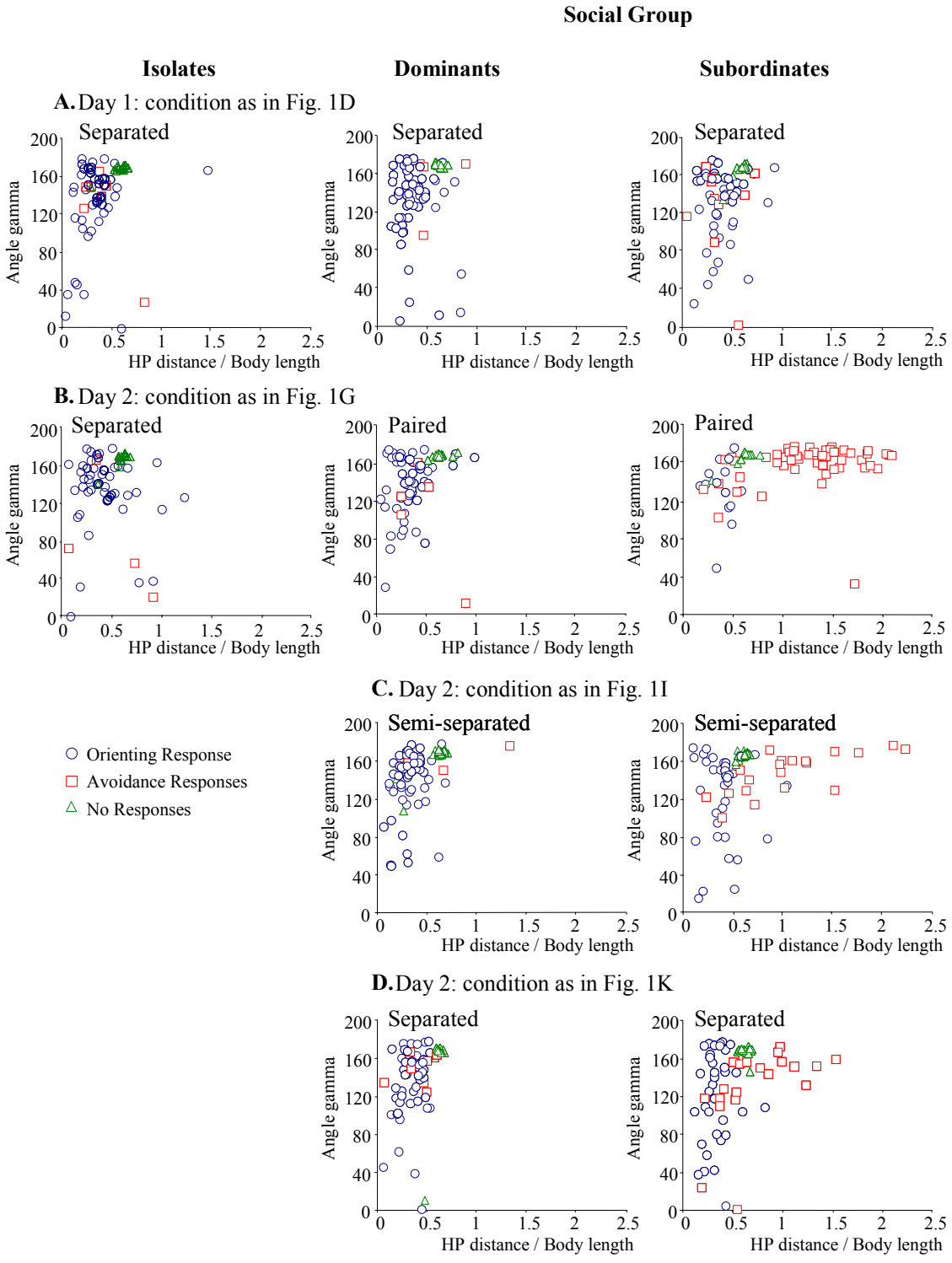
To visualize the difference between the Orienting and the Avoidance responses of the socially experienced and inexperienced animals, the positions of each animal before and after the stimulus were quantified (see Methods for details). Two related measures were used: the distance between the final head position and the site of the probe's touch on the animal's side, in body lengths (HP), and the angle γ between the animal's body axis and the direction from the body center to the site of the probe contact.

'No Responses' were characterized by low HP values and γ angles between 90^o and 180^o, whereas Orienting Responses were associated with similar HP values and a broader range of angles, between 0 and 180^o (Fig. 5). Avoidance Responses displayed much larger HP values and γ angles between 90^o and 180^o.

Isolate, dominant, and subordinate animals displayed similar responses on day 1 when tested while isolated (Fig. 4A). Their patterns of movement were also similar, clustering around low HP values and γ angles between 90^o and 180^o (Fig. 5A). A few animals of each social type displayed Avoidance or Orienting Responses characterized by low γ values. The movement pattern of the subordinates reflected the change in their responses when paired on day 2 (Fig. 4B). Subordinates displayed Avoidance Responses (red squares) with much larger HP values, extending to more than three body lengths (Fig. 5B). Most Avoidance Responses also had γ angles near 180^o, indicating that the animal

had moved forward, away from the probe. A smaller number had low γ values, indicating that the animal moved backward. When the animals were 'semi-separated' by an open divider, the responses of the social dominants did not change significantly (Fig. 4C, 5C), whereas the Avoidance responses of the subordinates were again characterized by large HP values and large γ angles, indicating forward movement away from the probe. Separation of the subordinates brought partial recovery of the original movement patterns; the HP distribution of Avoidance Responses was reduced from its highest values but still extended to greater values than when the subordinates were tested initially (Fig. 5D).

Fig. 5 The distribution of the HP distances and the γ angles of the Orienting (blue circle, Avoidance (red square), and No (green triangle) Responses of all animals tested. (**A~D**) Isolate and dominant animals displayed Orienting responses. While separated (**A**, $\mathbf{B_{isol}}$, and $\mathbf{D_{dom}}$), while paired ($\mathbf{B_{dom}}$), and while separated by an open divider ($\mathbf{C_{dom}}$), most isolate and dominant animals displayed Orienting Responses although some Avoidance Responses and No Responses were also produced. Note that the Avoidance Responses produced by the isolate and the dominant animals have small HP distance value (<1). (**C**) Subordinate animals displayed Orienting Responses when separated but they displayed Avoidance Responses when interacting with dominant opponents. The subordinate animals produced Orienting Responses while separated on day 1 ($\mathbf{A_{sub}}$) similar to the isolate and the dominant animals. While paired ($\mathbf{B_{sub}}$), the subordinate animals showed Avoidance Responses. Note that the Avoidance Responses produced by the subordinates have large HP distance value (1 < HP distance value < 2.5). In a semi-separated condition ($\mathbf{C_{sub}}$), some subordinate animals showed Orienting Responses whereas other subordinate animals continued to show Avoidance Responses and large HP values. While separated on day 2 ($\mathbf{D_{sub}}$), most subordinate animals showed Orienting Responses, while three subordinate animals produced Avoidance Response with large HP values (>1).


Social Group
Isolates
Dominants
Subordinates
A. Day 1: condition as in Fig. 1D
B. Day 2: condition as in Fig. 1G
C. Day 2: condition as in Fig. 1I
D. Day 2: condition as in Fig. 1K
Separated
Paired
Semi-separated
Angle gamma
HP distance / Body length
Orienting Response
Avoidance Responses
No Responses

IV. DISCUSSION

Both the nervous system and behavior of crayfish depend on the animal's social status. Serotonergic modulation of the excitability of the command neurons for escape and the threshold for escape behavior depend on the social status of crayfish (Yeh et al., 1996a, 1996b, 1997; Krasne et al., 1997; Teshiba et al., 2001), and crayfish display agonistic behaviors typical of their social status (Goessmann et al., 2000; Herberholz et al., 2001). Dominant and subordinate animals also differ in their willingness to engage in other non-social behaviors, such as shelter construction, in the presence of a social partner (Herberholz et al., 2003). Subordinate animals are inhibited from digging in the presence of a dominant partner, while the dominant's burrowing activity is increased. The inhibition was conditional upon the near presence of the dominant, but also appeared to linger after the subordinate had been isolated.

Previous reports indicated that the response of a crayfish to an unexpected touch depended on the animal's size (Nagayama et al., 1986) or dominance status (Bovbjerg, 1953). The types of escape reflex circuitries to be activated were dependent on social status of crayfish under the threat of unexpected attack, while the excitability of the lateral giant escape reflex was independent of social status when the threat was removed (Krasne et al., 1997). Here I have found that a crayfish's response to an unexpected touch depends on the animal's social context as well as its social status. The response of a social subordinate in the presence of its dominant partner differed from its response when alone. When kept apart from their partner by a closed barrier, the social status or experience of the animal had no effect on its responses, which were identical to those of

socially isolated animals. Isolates and separated dominants and subordinates all turned to confront the source of the unexpected touch (Fig. 4A, 5A, 5B). However, when subordinates were tested while in partial contact with their dominant partners (i.e., 'semi-separated), their patterns of responses differed from when tested alone (Fig. 4C, 4E, 5C). These differences increased dramatically when the subordinates were tested in the presence of their dominant partner: instead of turning to confront the unexpected touch, they moved away (Fig. 4B, 4E, 5B). It is apparent, therefore, that as in the case of burrowing behavior (Herberholz et al., 2003), the social context of being subordinate in the presence of the dominant partner determined the change in the subordinate's response to the stimulus.

Subsequent tests showed that the change in the subordinate's response brought about by being tested while paired persisted to a small degree for some hours (Fig. 4E). Tests while semi-separated (Fig. 4C, 5C) and while alone (Fig. 4D, 5D) revealed that the subordinates' behavior was not restored to that of the earlier isolated condition (Fig. 4A, 5A) five hours after the tests while paired. However, 24 hours after previous pairing experience in semi-separated condition, subordinates' behavior appeared to be fully restored to that of the earlier isolated condition (data not shown).

These results suggest that the effect of the paired context in which the unexpected touch was received lingers for some hours after the event. This effect may be a state change, similar to fear, or it may be a memory (i.e., a specific association) of having received the earlier unexpected touches in the presence of the dominant. When the subordinates were tested while semi-separated from their dominant partners, this state or memory may have been strengthened by olfactory stimuli from the dominant. When the

subordinates were finally tested while isolated from their dominant partners, the response pattern showed some influence of the earlier paired context in which the animals were tested.

The persistence of the change in the subordinate's behavior when the dominant was absent is similar to the lingering inhibitory effect of the dominant on the subordinate crayfish's burrowing activity (Herberholz et al., 2003). In both these cases, it is not clear whether this persistence represents a specific memory of a more generalized change in behavior, like fear. Crayfish, like other crustaceans, can learn specific associations (Krasne, 1974). Furthermore, gradual increase, although small, in the average frequencies of No Responses between the dominant and the subordinate animals after the recent pairing experience suggests that the two animals may have habituated to the repeated touch stimuli. Several studies have demonstrated the ability of crayfish to learn to recognize crayfish in the process of dominance hierarchy formation and maintenance (Bovbjerg, 1953; for individual recognition: Lowe, 1956; recognition of aggressive state: Copp, 1986; Status recognition: Zulandt-Schneider et al., 1999), although what is recognized and when the animal learns are not completely understood.

The complete neural mechanisms of the different behavioral responses to unexpected lateral touch are unknown, but some elements have been identified. Bilateral pairs of serotonergic neurons in the abdomen and thorax of crayfish receive both excitatory and inhibitory inputs in response to a lateral touch of the rostral portion of the abdomen, but the mix of excitation and inhibition depends on the social status of the animal (Drummond et al., 2002). The ipsilateral 5-HT neurons of isolate and dominant crayfish were excited by a unilateral touch while the contralateral 5-HT neurons were

inhibited. The same neurons in subordinate crayfish were symmetrically excited in several crayfish and inhibited in as many others; asymmetric responses did not occur. These 5-HT neurons have been found to modulate walking leg reflexes, which also display differences between dominant and subordinate animals (Issa et al., 2004). It is tempting to link the asymmetric neuronal responses of isolate and dominant crayfish to their orienting responses described here, and the symmetric excitatory or inhibitory neuronal responses of subordinate crayfish to their symmetric avoidance responses. Future studies will determine whether these correlations between neuronal responses and behavior are indicative of underlying causal mechanisms.

Chapter 2. Social Status and Neurogenesis:

Changes in Brain Neurogenesis after A Dominance Hierarchy Formation

CHAPTER 2

Social Status and Adult Neurogenesis:

CONTENTS

I. INTRODUCTION

Since Altman (1962) reported the occurrence of neurogenesis in the adult rat brain, many studies have demonstrated neurogenesis in the hippocampus and olfactory bulb of adult vertebrates including humans (for a review, Ming and Song, 2005). Many factors regulate the level of such neurogenesis, including exercise (van Praag et al., 1999), stress (Gould et al., 1997, 1998; Pham et al., 2003), environmental richness (Kempermann et al., 1998; Brown et al., 2003), learning and memory formation (Gould et al., 1999; Shors et al., 2001), and enriched odor inputs (Rochefort et al., 2002). Recently, dominance hierarchy formation – a pattern of repeated agonistic interactions with an expected outcome of winner and loser – has been shown to influence neurogenesis in the hippocampus but not the olfactory bulb (Kozorovitskiy and Gould, 2004; Pravosudov and Omanska, 2005). Persistent neurogenesis also takes place in adult arthropods, including in the brains of insects (Cayre et al., 2002), and in the central and peripheral olfactory system of juvenile and adult crustaceans (Schmidt, 1997; Harrison et al., 2001; Sandeman and Sandeman, 2000, 2003; reviewed by Schmidt, 2002; Beltz and Sandeman, 2003). In the brains of insects and crustaceans, the levels of juvenile / adult neurogenesis are influenced by many factors, such as hormones and polyamines (Cayre et al., 1994), serotonin levels (Beltz et al., 2001), sensory inputs (Hansen and Schmidt, 2001; Scotto-Lomassese et al., 2002), environmental richness (Sandeman and Sandeman,

2000; Hansen and Schmidt, 2004), circadian rhythm (Goergen et al., 2002), and season (Hansen and Schmidt, 2004).

Crayfish are excellent models for examining the effect of social interactions on olfactory neurogenesis for several reasons. First, new neurons are continuously added to the populations of local and output olfactory interneurons and of olfactory receptor neurons of crayfish throughout their lives (Sandeman et al., 1998; Schmidt and Harzsch, 1999; for a review, Sandeman and Sandeman, 2003). Second, crayfish form dominance hierarchies quickly and stably, leading to changes in their physiology and social and non-social behaviors (Bovbjerg, 1953; Lowe, 1956; Issa et al., 1999; Song et al., 2000; Edwards et al., 2003; Herberholz et al., 2003). Third, crayfish use chemical signals during social interactions and in forming and maintaining dominance hierarchies (Zulandt Schneider et al., 1999, 2001; Breithaupt and Eger, 2002). Although many factors are known to influence olfactory neurogenesis in crayfish, pair-wise social experience has not been examined.

In this chapter, the life-long proliferation zones in cell clusters 9 and 10 of crayfish brain are re-defined as 'comma'-shaped, and morphological characteristics of the 'comma-shaped proliferation zone are examined with respect to the location, size, and shape of newborn neuronal percursors. In addition, the influence of social interactions on neurogenesis was examined in the central olfactory system of the crayfish *Procambarus clarkii*. This chapter determined how the proliferation and survival of cells in the clusters containing local and projection olfactory interneurons are influenced by up to 14 days of social pairing. The level of cell proliferation is similar in both dominant and subordinate

animals, while the level of cell survival is higher in dominant animals compared to subordinates.

II. MATERIALS AND METHODS

II.1. Animal Preparation

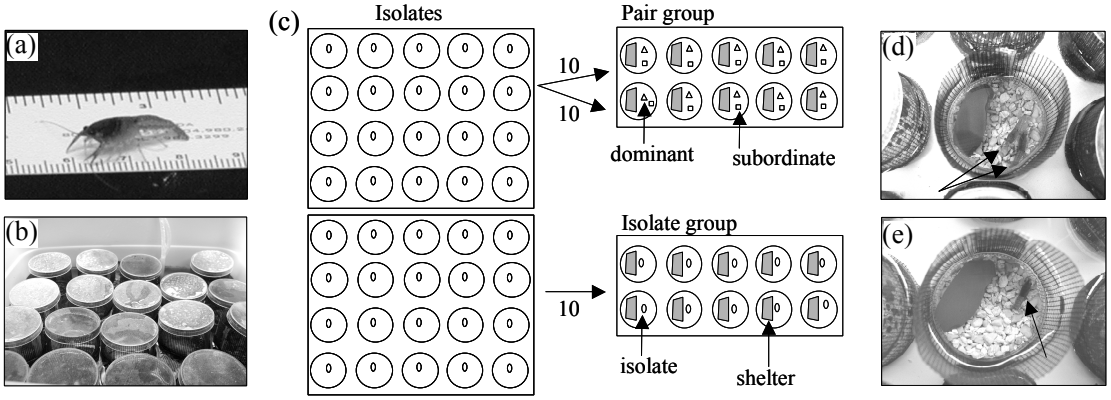
All animals used in this study hatched in the laboratory from mother crayfish, *Procambarus clarkii*, purchased from Atchafalaya Biological Supply (Raceland, LA). Hatchlings were individually isolated in small cages when they became free-swimming (a in Fig. 6A). These cages were cylindrical (5.5 cm in diameter, 7.0-7.5 cm in height) with plastic mesh walls on which animals could climb. A lid was placed on top to prevent escape (b in Fig. 6A). Twenty cages were placed together in a plastic aquarium (33 cm x 14.5 cm x 29 cm, 14 L); thus individual crayfish were physically, but not chemically, isolated (b in Fig. 6A). All animals were reared for 5-9 weeks in order to control the developmental stage as well as previous living experience. To indicate the developmental stage of the crayfish used in this study, which had miniature adult form and grew by molting approximately once a week, the term 'juvenile' was used in the text following the terminology in Holdich (1992). Juvenile crayfish were reared in constantly aerated water, in a 12:12 light:dark cycle, fed twice a week with a piece of turkey meat (approximately 0.1 cm^3, 0.6 mg) and a piece of carrot of similar size in the late afternoon during isolation, and provided with water plants (*Elodea* sp.) *ad libitum*.

II.2. Preparations for Isolate and Paired Animals

To create conditions wherein crayfish form a stable dominance hierarchy, pairs of previously isolated crayfish that hatched from the same mother crayfish were selected based on a difference of 10-20% in wet body weight, ranging from 0.02-0.20 g at the beginning of pairing and from 0.04-0.20 g at the end of pairing, and placed together in a small cage (Pair group; c, d in Fig. 6A). Body size is generally thought to be the best predictor of fight outcomes in crayfish (Bovbjerg, 1953; Lowe, 1956). Pairs were created regardless of sex because the crayfish in this study were not sexually mature and because at this stage, sex does not affect the outcome of social encounters (Rhodes and Holdich, 1979). Only one shelter was provided in each cage to promote interactions between the animals (c, d, e in Fig. 6A). The shelter was a piece of broken clay pot with enough curvature for an animal to reside comfortably under it. Additionally, ten previously isolated animals were individually placed in the same type of cages, and ten cages were placed together in a same-sized plastic aquarium (Isolate group; c, e in Fig. 6A). Although chemical signaling between individual animals in the Isolate groups or between pairs in the Pair groups was not controlled, the Pair groups and the Isolate groups were in different aquaria so that the Isolate groups were not exposed to the chemicals of the Pair groups.

Fig. 6 Experimental design. **A.** Animal setup. **(a)** A juvenile crayfish, 2.0 cm from rostrum to telson. **(b)** Individual cages in an aquarium. Each cage is 5.5 cm in diameter. **(c)** Diagrams illustrating setup for animals in the Isolate and Pair groups. Rectangles and circles represent plastic aquaria and cages, respectively. Small ovals in each circle in the Isolate groups represent animals in isolation, while small triangles and small squares in each circle in the Pair group represent dominant and subordinate animals, respectively. Each gray object inside a circle represents a shelter. For the Pair group, 20 animals were transferred to new cages, two animals per cage, and only one shelter was provided in each cage. For the Isolate group, ten animals were individually transferred to new cages and a shelter was provided in each new cage. **(d)** Two animals in the Pair group (arrows) in a cage with one shelter. **(e)** One animal in the Isolate group (arrow) in a cage with one shelter.

A. Animal setup



II.3. Preparations for Cell Proliferation and Survival Tests

The Pair and Isolate groups were further divided into five groups to test the effect of dominance hierarchy formation on cell proliferation and cell survival in the brain. When two unacquainted crayfish meet, bouts of intense agonistic interactions arise immediately, and in less than half an hour, they establish a winner-loser relationship, a form of dominance hierarchy (Issa et al., 1999; Edwards et al., 2003). Intense agonistic interactions can last for several hours afterwards; however, as days pass, agonistic interactions between the acquainted crayfish become less intense and less frequent (Issa et al., 1999; Edwards et al., 2003). Thus, three time points were chosen to examine whether the dominance hierarchy formation influences cell proliferation and survival: acute (1 day), intermediate (7 days), and long-term (14 days).

For the cell proliferation test, same-age siblings that were individually reared since hatching were placed in cages in pairs on the same day and reared together for 1, 7, or 14 days, then immersed in BrdU solution (1 mg BrdU / 1 ml water) for 24 hr, and sacrificed (Fig. 7A). Isolate animals in each of the cell proliferation test groups were same-age siblings and treated the same except that they remained isolated for an additional 1, 7, or 14 days in lieu of pairing.

For the cell survival test, two groups of animals were immersed in BrdU solution for 24 hr at the beginning of pairing and sacrificed after 7 days or 14 days of pairing (Fig. 7B). Isolate animals in each of the cell survival test groups were same-age siblings and treated the same except that they remained isolated for 7 or 14 days.

Cell proliferation was measured in two ways. *In vivo* labeling with BrdU was used to measure DNA replication. BrdU (5-bromo-2′-deoxyuridine) is a thymidine

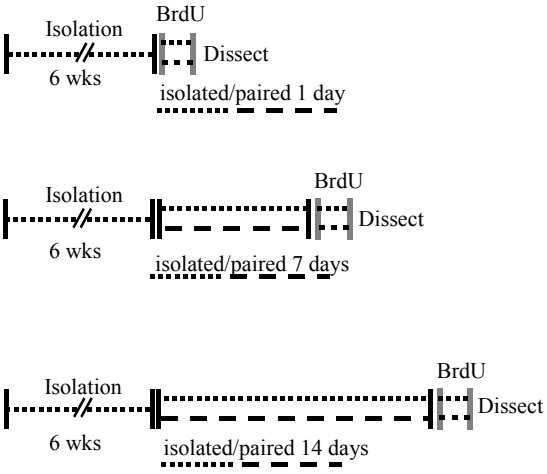
analog that is incorporated into DNA during the S phase of the cell division cycle (Fig. 7C). Phospho-histone-3 (H3P) was used as a mitosis marker to visualize cells in their mitotic (M) phase (Fig. 7C) (Harzsch et al., 1999).

To determine the level of cell proliferation in the animals used in the cell survival test at the beginning of pairing period, two age-control groups were prepared, one each for the 7- and 14-day survival groups. Animals in each age-control group were same-age siblings of those in the corresponding cell survival test groups and were immersed in BrdU solution for 24 hr in isolation on the same day but sacrificed immediately (Fig. 7B).

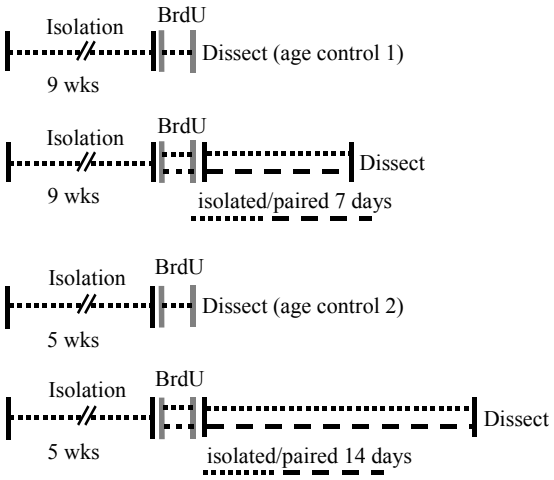
To examine the distribution of newborn cells within comma-shaped proliferation zone, all animals in the 1 day, 7 or 14 days of cell proliferation test groups were pooled as a 1 day pulse-chase group. All animals in the 7 and 14 days cell survival test groups were pooled as 7 and 14 days pulse-chase groups, respectively. This pooling was done only for morphological analysis of newborn cells in cell clusters 9 and 10, such as the proportion, number, size, and shape of BrdU+ nuclei. For examination of the effect of social status on cell proliferation and cell survival, tested animals are grouped into 1 day, 7 days, and 14 days cell proliferation test groups and 7 days and 14 days cell survival test groups.

Fig. 7 **A-B.** Protocols for cell proliferation test and cell survival test. Following the protocol in A and B, the animals in the Isolate and Pair groups were treated for cell proliferation and cell survival tests. A dotted line indicates an isolated condition; a dashed line indicates a pairing condition; gray bars indicate immersion of animals in BrdU solution for 24 hr. **A.** Cell Proliferation Test. Animals were isolated for 6 weeks, paired or isolated for 1, 7, or 14 days, immersed in BrdU for 24 hr at the end of pairing period, and sacrificed. **B.** Cell Survival Test. All animals were isolated either for 5 or 9 weeks, paired or isolated for 7 or 14 days, immersed in BrdU for 24 hr at the beginning of pairing period, and sacrificed. A separate group of animals (age-control groups 1 and 2) was treated similarly (isolated for 5 or 9 weeks, immersed in BrdU for 24 hr, and sacrificed immediately) but without any pairing. The age-control groups show the level of cell proliferation at the end of the isolation period, while the animals in the Isolate group show the level of cell survival after 7 days or 14 days of isolation. **C.** Diagram of the cell division cycle. BrdU, a thymidine analog, labels cells in the S phase during which DNA replication occurs. H3P, a mitosis marker, labels cells in the M phase during which histone 3, a protein mediating the folding of DNA, is phosphorylated.

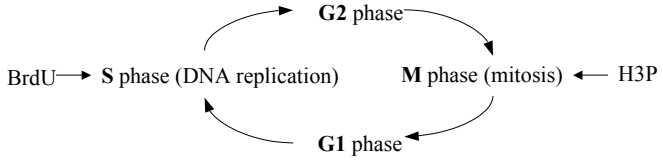
A. Cell Proliferation Test



B. Cell Survival Test



C. Cell Division Cycle



II.4. Behavioral Observation and Feeding

Animals were individually marked at the beginning of each experiment. When one animal in a pair molted, both animals in the pair were marked again. Regardless of their social status, most animals used in 7-day cell proliferation and survival tests did not molt, while a few molted once. In 14-day cell proliferation and survival tests, most animals molted once or twice, while a few did not molt.

When two isolated juvenile crayfish were placed together in a cage with one shelter, bouts of intense fighting occurred immediately, and within less than an hour, a dominant-subordinate relationship formed between the two animals (Bovbjerg, 1953; Issa et al., 1999).

Control of feeding during the experiment is important because nutritional state directly influences the rate of body growth and probably influences the level of neurogenesis. Feeding was controlled by giving each animal similar-sized pieces of turkey meat in the late afternoon of each day to help keep all animals on the same circadian cycle of feeding (Goergen et al., 2002). This food was in addition to water plants (*Elodea* sp.) available *ad libitum*. Behavioral observations were also made during each feeding to ensure that each member of the pair consumed its piece of turkey meat. A piece of turkey meat was given first to the dominant animal, which often approached the forceps holding the turkey meat, and then to the subordinate animal while the dominant partner was eating its piece. In this way, subordinate animals received the same amount of protein-rich food as the dominants, and avoided yielding their share to the dominant animals. When the animals were quiescent and their relative social status

was unclear, presentation of a piece of turkey induced social interactions that revealed the social status of each animal.

II.5. Brain Histology

Animals in each group were removed from the cage and immediately placed in ice-chilled water for 10-15 min, after which their brains were dissected out in cold saline, fixed overnight in 4% paraformaldehyde, rinsed in 0.1 M phosphate buffer (PB), and stored at 4°C in PB with 0.1% sodium azide. Animals were analyzed for neurogenesis only if they were from a pair that formed a stable dominance hierarchy and if both of their antennular lateral flagella, the olfactory organs of the crayfish, remained intact. Six animals (2 dominants and 4 subordinates) out of the total 28 pairs were excluded from the analysis due to damage to their antennular lateral flagella. The partners of those six animals were included in the analysis if their antennular lateral flagella remained intact.

II.6. Immunocytochemistry

Brains from cell proliferation and cell survival test groups were embedded in 14% gelatin solution (1.4 g gelatin / 10 ml 0.1 M PB), fixed overnight in 4% paraformaldehyde at 4°C, rinsed (4x30 min in 0.1 M PB), and horizontally sectioned at 70-100 μm thickness using a vibrating microtome (VT 1000 S, Leica: Wetzlar, Germany). Brain sections were incubated in 2 M HCl for 30 min at room temperature, rinsed (3x30 min in 0.1 M PB), incubated for 4 hr in blocking solution (5% normal goat serum, 1% bovine serum albumin, 0.1% sodium azide, and 0.3% Triton X-100 in 0.1 M PB), and incubated overnight at room temperature in a mixture of two primary antibody

solutions (monoclonal mouse anti-5-bromo-2′-deoxyuridine, prepared according to package instructions: RPN 202, Amersham Bioscience, Buckinghamshire, UK; and polyclonal rabbit anti-phospho-histone-3 (H3P), 1:200 dilution; Upstate, Waltham, MA). The sections were then rinsed (4x30 min in 0.1 M PB), incubated in a mixture of secondary antibodies (Alexa fluor 488, goat anti-mouse Ig G; Texas Red, goat anti-rabbit Ig G; each diluted 1:200; Molecular Probes, Eugene, OR) for 2-3 hr, rinsed (4x30 min in 0.1 M PB), and mounted in a 1:1 mixture of glycerol and PB. Images of brain sections were collected using a confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY). Stacks of individual images were reconstructed using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

In addition to the cell proliferation and cell survival test groups, four brains of approximately 4-week old juvenile crayfish were dissected out immediately after 24 hr immersion in BrdU solution (1 mg / ml), fixed overnight in 4% paraformaldehyde, and rinsed 6 x 1 hr in PBTX (0.1M PB with 0.25% Triton X-100). After dehydration and rehydration through an ethanol series, the brains were incubated overnight in mouse monoclonal anti-BrdU, rinsed 6 x 1 hr in PBTX, incubated in rat monoclonal anti-tubulin (YOL 1/34 microtubule marker, Abcam, Cambridge, MA; diluted 1:200), rinsed 6 x 1 hr in PBTX, and then incubated for 4 hrs in a mixture of Cy3 goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488 goat anti-rat (Molecular Probes, Eugene, OR) each diluted 1:50. The brains were then rinsed 6 x 1 hr in PB, incubated in a 0.002% solution of Hoechst 33342 for nuclear staining, rinsed again in PB, dehydrated through an ethanol series, and mounted ventral side up in methyl salicylate.

Additionally, four adult crayfish of both sex, approximately 4-5 cm in body size from head to telson, were immersed in BrdU solution (1 mg / ml) for 24 hours, injected with 3 ml of dextran-linked Texas red (0.05 % w/v, Molecular Probes, Eugene, OR) at ventral sinus, and immediately sacrificed. Their brains were dissected out and fixed overnight in 4% paraformaldehyde, rinsed 4x30 min in PB, embedded in 14% gelatin solution (1.4 g gelatin / 10 ml 0.1 M PB), fixed overnight in 4% paraformaldehyde at 4°C, rinsed (4x30 min in 0.1 M PB), and horizontally sectioned at 70-100 μm thickness using a vibrating microtome (VT 1000 S, Leica: Wetzlar, Germany). Brain sections were incubated in 2 M HCl for 2 hr at room temperature, rinsed (3x30 min in 0.1 M PB), incubated overnight in mouse monoclonal anti-BrdU, rinsed 4x30 min in PBTX (0.1M PB with 0.3% Triton X-100), incubated in secondary antibodies (Alexa fluor 488, goat anti-mouse Ig G diluted 1:200; Molecular Probes, Eugene, OR) for 4 hr, rinsed (4x30 min in 0.1 M PB), and mounted on slide using Gel/Mount (Biomeda corp., Foster city, CA).

II.7. Cell Counting Analysis

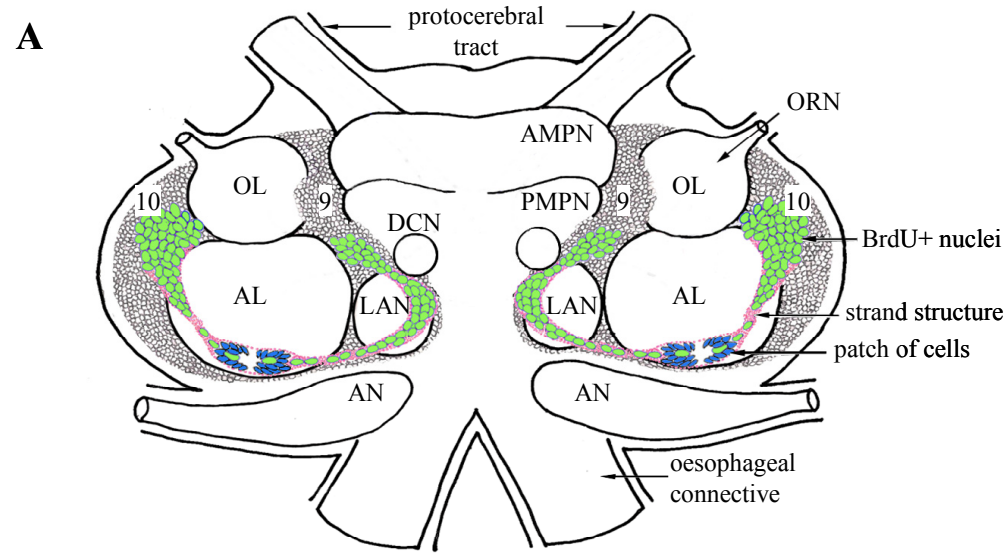
Stacks of confocal images were imported into Image-Pro Express software (Media Cybernetics, Silver Spring, MD). Unlike the vertebrate olfactory bulb, which has within it the somata of olfactory interneurons organized into layers within the neuropil, the crayfish brain has two large paired lobes, the olfactory lobe and the accessory lobe, and the somata of its interneurons are positioned outside of these neuropils: cell cluster 9 contains the cell bodies of olfactory local interneurons, and cell cluster 10 contains the cell bodies of olfactory projection interneurons (Fig. 9A). All individual BrdU-labeled (BrdU+) cells in cell clusters 9 and 10 were traced and counted in a blind fashion

according to the following criteria. BrdU+ cells associated with clusters 9 and 10 are typically grouped in a 'comma' shape, consisting of a 'head' and 'tail' surrounded by a 'strand structure', as it is described in the Results section (Fig. 9B). BrdU+ cells in the head and tail parts of cell clusters 9 and 10 were counted separately. For the head part, BrdU+ cells within the boundaries of cell clusters 9 and 10 were counted, excluding BrdU+ cells with nuclei of lenticular shape (see dotted circles in Fig. 9B). For the tail part, which included the patch of cells near the posterior AL, BrdU+ cells within the strand structure were counted but BrdU+ cells with nuclei of lenticular shape were excluded (see dashed circles in Fig. 9B, 10A-10B). BrdU+ cells were not counted when they were outside of cell clusters 9 and 10 and the strand structure or when they were in other areas of the brain, i.e., on the borders or on top of neuropils (arrowheads in Fig. 10C-10D). This way, all individual BrdU+ nuclei appearing within the comma-shaped proliferation zone were counted, and thus there was no need to apply stereological estimation.

Six animals (2 dominants and 4 subordinates) out of the total 28 pairs had damage to one of the antennular lateral flagella of the antennules. BrdU+ cells in the brain ipsilateral and contralateral to the damaged flagellum were counted but not included in the analysis because damage to this chemosensory organ is known to influence neurogenesis (Sandeman et al., 1998; Hansen and Schmidt, 2001; Sandeman and Sandeman, 2003). The partners of those six animals were included in the analysis if their lateral flagella remained intact. The median number of BrdU+ cells in the brains having partial damage to one lateral flagellum was generally lower than the median number of BrdU+ cells in the brains having two intact lateral flagella (see the numbers in

parenthesis in Table 1). In three preparations, damage to one side of the brain occurred during handling and processing, the number of BrdU+ cells in the intact side of the brain tissues was counted and its number was doubled assuming a symmetry of neurogenesis in both sides of the brain. This is justifiable because of a high correlation between numbers of BrdU+ cells in the left and right sides of brains of undamaged animals in this study (linear regression, $R_{cl\ 9} = 0.945$ and $R_{cl\ 10} = 0.794$ when R = 1.0 represents symmetry). H3P-labeled (H3P+) cells in cell clusters 9 and 10, mostly colocalized with BrdU, were counted in the same manner as described above.

Fig. 8 'Comma-shaped' proliferation zone. **A.** Diagram of juvenile crayfish brain identifying the neuropils and cell clusters (modified from Sandeman et al., 1992) as well as the neurogenic areas in the deutocerebrum (this study). Primary sensory inputs from olfactory receptor neurons (ORNs) in olfactory organs enter the olfactory lobe (OL). The cell bodies of the olfactory local and projection interneurons comprise cell clusters 9 and 10, respectively. Groups of BrdU+ cells (shown in green) appear in cell clusters 9 and 10 and lines of BrdU+ cells, surrounded with a strand structure (shown in violet), meet at a patch of cells (shown in blue) near the posterior AL. 9 and 10, cell clusters 9 and 10; AL, accessory lobe; AMPN, anterior median protocerebrum neuropil; AN, antennal neuropil; DCN, deutocerebral commissure neuropil; LAN, lateral antennular neuropil; OL, olfactory lobe; ORN, olfactory receptor neuron, PMPN, posterior median protocerebrum neuropil. **B.** A juvenile crayfish brain showing robust cell proliferation in cell clusters 9 and 10. Each dotted circle (orange for cluster 9 head part and white for cluster 10 head part) indicates regions where a ball of BrdU+ cells is present within the cluster boundary. Each dashed circle indicates a region around the LAN (orange for cluster 9 tail part) or around the posterior AL (white for cluster 10 tail part) where lines of BrdU+ cells appear. Scale bar = 50 μm.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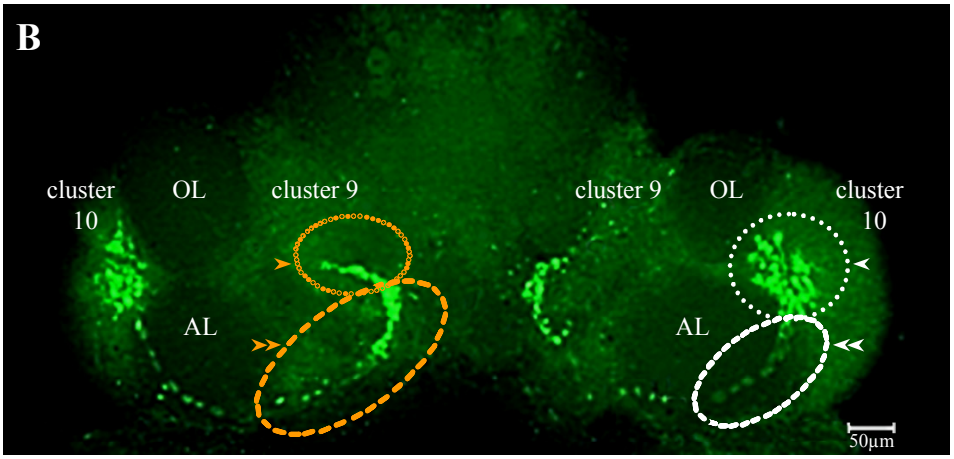
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II.8. Cell Distribution Analysis: Location, Size, and Shape

ImagePro Express was used to measure the cross-sectional area and the perimeter of BrdU+ nuclei. The Feret diameter of the nuclei of BrdU+ cells were calculated, the Feret diameter ($D = \text{sqrt}(4 \times \text{area} / \pi)$) and roundness ($R = 4\pi \times \text{area} / \text{perimeter}^2$ where R of a perfect circle is 1.0) was calculated. These results are presented after pooling each test group according to its pulse-chase context. This pooling was justifiable because there were no significant statistical differences across the social status of animals in most groups of cell proliferation and cell survival tests.

II.9. Statistical Analysis

The numbers of BrdU+ cells and H3P+ cells in isolate, dominant, and subordinate animals were subjected to non-parametric one-way ANOVA (Kruskal-Wallis test) using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) while treating the three types of animals independently. When this analysis revealed a statistically significant difference, non-parametric two-tailed t-tests (Mann-Whitney test) were used to determine which groups differed. Separate analyses were run for counts of the entire comma of BrdU+ cells, its head part, and its tail part, for both cluster 9 and cluster 10.

To determine if the social status of an animal affected the number of BrdU+ cells above and beyond possible effects of body growth rate, analysis of covariance (ANCOVA) was used to control for differences in the body growth rates of the animals over the experimental period. For this analysis, the univariate General Linear Model procedure of SPSS 12.0 for Windows (SPSS, Chicago, IL) was used.

III. RESULTS

III.1. Morphological Study of Proliferating and Surviving Olfactory Interneuronal Precursors

III.1.1. Organization of Crayfish Brain around Clusters 9 and 10

The brain of the crayfish is dominated by the deutocerebrum with its antennular and olfactory processing regions – the olfactory lobes (OLs), accessory lobes (ALs), lateral antennular neuropils (LANs), and deutocerebral commissure neuropils (DCNs) (Fig. 8A). The OL receives input from olfactory receptor neurons (ORNs) that project centrally from aesthetasc sensilla on the lateral flagellum of the antennule, the chemosensory organ. The LAN receives input from chemoreceptor neurons and mechanoreceptor neurons that are housed in the non-aesthetasc sensilla on the lateral and medial flagella of the antennule (Schmidt and Ache, 1996a, 1996b). The DCN receives inputs from the deutocerebral commissure and from the medial protocerebrum (Sandeman et al., 1992; Mellon and Alones, 1993; Schachtner et al., 2005). The AL receives no direct sensory input, but it is highly connected to the OL and receives higher-order multisensory inputs (Sandeman et al., 1995; Sullivan and Beltz, 2001).

The OL and AL contain olfactory local interneurons (OLNs) and olfactory projection interneurons (OPNs) whose cell bodies are in cell clusters 9 and 10, respectively (Fig. 8A). In juvenile crayfish, cell cluster 9 extends posteriorly much farther in the ventral regions than in more dorsal regions; it is medial to the AL and OL

and its most posterior region is ventral to the LAN. Cell cluster 10 is lateral to the OL and AL throughout the ventral-dorsal plane (Fig. 8A). The axons of the OPNs exit the OLs and ALs and form an output pathway, the olfactory globular tract, which crosses the midline of the brain and projects bilaterally to the terminal medulla and the hemiellipsoid bodies in the eyestalks (Sullivan and Beltz, 2001).

III.1.2. 'Comma' Shape Proliferation Zone in Clusters 9 and 10

After crayfish were exposed to BrdU, numerous BrdU+ cells were present in clusters 9 and 10 (Fig. 8), as has been reported previously for juvenile and adult crayfish (Sandeman and Sandeman, 2000; for a review, Sandeman and Sandeman, 2003). Previous studies with laboratory reared adult crayfish, report that the number of BrdU+ cells in cluster 9 is generally lower, approximately one sixth (Sandeman and Sandeman, 2000) to zero (Schmidt and Harzsch, 1999) than the number in cluster 10. In cell clusters 9 and 10 in the brain of the juvenile animals examined in this study, a group of BrdU+ cells in each cluster appeared as a comma shape in all animals examined in this study regardless of sex, molt stage, size, and social status of an animal (Fig. 8). The 'head' part of the comma was located within the cell cluster boundary, and the 'tail' part of the comma extended from the head beyond the cluster boundary (see dotted circles in Fig. 8B). In cell cluster 9, the tail part of the comma curved around the LAN and extended toward the posterior AL (orange dashed circle in Fig. 8B). In cell cluster 10, the tail part of the comma extended medially and ventrally across the ventral surface of the AL (white dashed circle in Fig. 8B). In adult crab, BrdU+ cells similar to the tail part have been

described in cell cluster 10 as “string-like” (Schmidt, 1997). Earlier studies done with crayfish and lobsters reported only the head part of the comma as a life-long proliferation zone (Sandeman et al., 1998; Harzsch et al., 1999). Thus, neurogenesis within the comma-shaped neurogenic area in cell cluster 9, which are more robust than those in cell cluster 10, have never been described in the literature before. The younger the animal was, the more prominent was the comma shape of the group of BrdU+ cells in both cell clusters 9 and 10.

The lenticular nuclei appeared in clusters 9 and 10, on the borders of neuropil, and along the tracts of neurites throughout the crayfish brain. The lenticular nuclei differ from the round shape of most BrdU+ nuclei in the comma and from the shape of the nuclei in unlabeled mature neurons in clusters 9 and 10. They may be glia (Harzsch et al., 1999).

No neuron-specific markers are currently known for crustaceans; nonetheless, glia-like cells were identified based on their possessing nuclei of lenticular shape and their location on the border of neuropils (Harzsch et al., 1999), and these were not included in the counts of BrdU+ cells in this study. Although some of BrdU+ cells within the comma-shaped proliferation zone might differentiate into glial cells, the organization of cell clusters 9 and 10 – composed of densely clustered cell bodies of olfactory interneurons (Fig. 8-9), suggests that ‘most’ BrdU+ cells are fated to become new olfactory interneurons. Other studies have shown that a majority of BrdU+ cells expresses neuronal phenotype after several months of survival time in juvenile crayfish and in adult lobsters (Schmidt, 2001; Sullivan and Beltz, 2005).

III.1.3. Strand Structure and A Patch of Cells

A strand structure surrounded the BrdU+ cells as well as H3P+ cells along the tail part of the comma (Fig. 9). The strand structure, being weakly positive to mitosis marker, was present along the BrdU+ cells in the tail part of the comma-shaped proliferation zone so that only the BrdU+ cells that were surrounded with the strand structure were counted (arrows in Fig. 9A-9B). Although it is not known whether BrdU+ cells within the strand structure are functionally related to BrdU+ cells within the boundaries of cell clusters 9 and 10, confocal images suggest their structural connection (Fig. 9). The strand structure was not associated with the vascular network in the brain, nor did the BrdU+ cells align with the network when the arteries were visualized via dye injection (Fig. 9C-9D). The strand structure was strongly labeled with anti-tubulin (Fig. 9E). The biological role of strand structure is unknown. The two tail parts from cell clusters 9 and 10, surrounded by the strand structure, joined at a patch of cells near the posterior AL (Fig. 9E-9F). Tubulin, the intracellular microtubule marker, labels microtubule aligned along the condensed DNA during mitosis (see arrow in Fig. 10). The strand structure and a patch of cells were also observed in the brains of adult crayfish (Fig. 11). The comma-shaped arrangement of BrdU+ and mitotic (H3P+) cells together with the strand structure and the patch of cells was present in clusters 9 and 10, regardless of the sex, molt stage, size, and social status of an animal (e.g., Fig. 8-11, Table 1). BrdU+ labeling was much more common than H3P+ labeling, with less than 5% of BrdU+ cells being H3P+ (Table 2).

To understand the morphological profile of the group of BrdU+ cells along the ventral-dorsal axis, diagrams were created based on observations (Fig. 12). The ventral-most layer contains the patch of cells and short lines of BrdU+ cells around the posterior LAN and around the posterior AL in cell clusters 9 and 10 that were surrounded by the strand structure (v1 in Fig. 12). More lines of BrdU+ cells around the anterior and medial LAN in cell cluster 9 and around the lateral AL in cell cluster 10, surrounded by the strand structure, emerge approximately 100 μm dorsal to the ventral surface of the brain (v2 in Fig. 12). A ball of BrdU+ cells in the head part of cell clusters 9 and 10 is situated 100-300 μm dorsal to the ventral surface of the brain (v3 in Fig. 12). The diagrams in Fig. 12 illustrate the whole profile of the group of BrdU+ cells, the strand structure, and the patch of cells with respect to the location of neuropils in the brain.

Fig. 9 Strand structure surrounding the comma-shaped proliferation zone. **A-F.** Stacks of confocal images taken at 0.5 μm interval. **A.** Lines of BrdU+ cells (green) in the tail part of cluster 9 are surrounded by a strand structure faintly labeled with anti-H3P (red, arrow). BrdU+ cells outside the strand (arrowheads) were not included in the counts of labeled cells. Asterisk indicates H3P+ (mitotic) cells. Scale bar = 50 μm. **B.** Lines of BrdU+ cells (green) in the tail part of cluster 10 are surrounded by a strand structure faintly labeled with anti-H3P (red, arrow). A BrdU+ cell on the border of AL (arrowhead) was not included in the counts of labeled cells. Asterisk indicates H3P+ (mitotic) cells. Scale bar = 50 μm. **C-D.** Lines of BrdU+ cells (green) in clusters 9 and 10 with respect to the brain vascularization (red). Scale bar = 50 μm. **E.** Strand structure, labeled with anti-tubulin (violet, arrows), surrounds lines of BrdU+ cells (green) from clusters 9 and 10, and a patch of cells (arrowhead). Scale bar = 50 μm. **F.** High-resolution image of the patch of cells (green, BrdU; blue, nuclear staining; violet, strand structure labeled with anti-tubulin) indicated by an arrowhead in (**E**). The patch of cells and a few BrdU+ cells are surrounded by a strand structure. Scale bar = 50 μm. Images of anti-tubulin staining (**E-F**) were kindly provided by Ms. Laurel M. Johnstone.

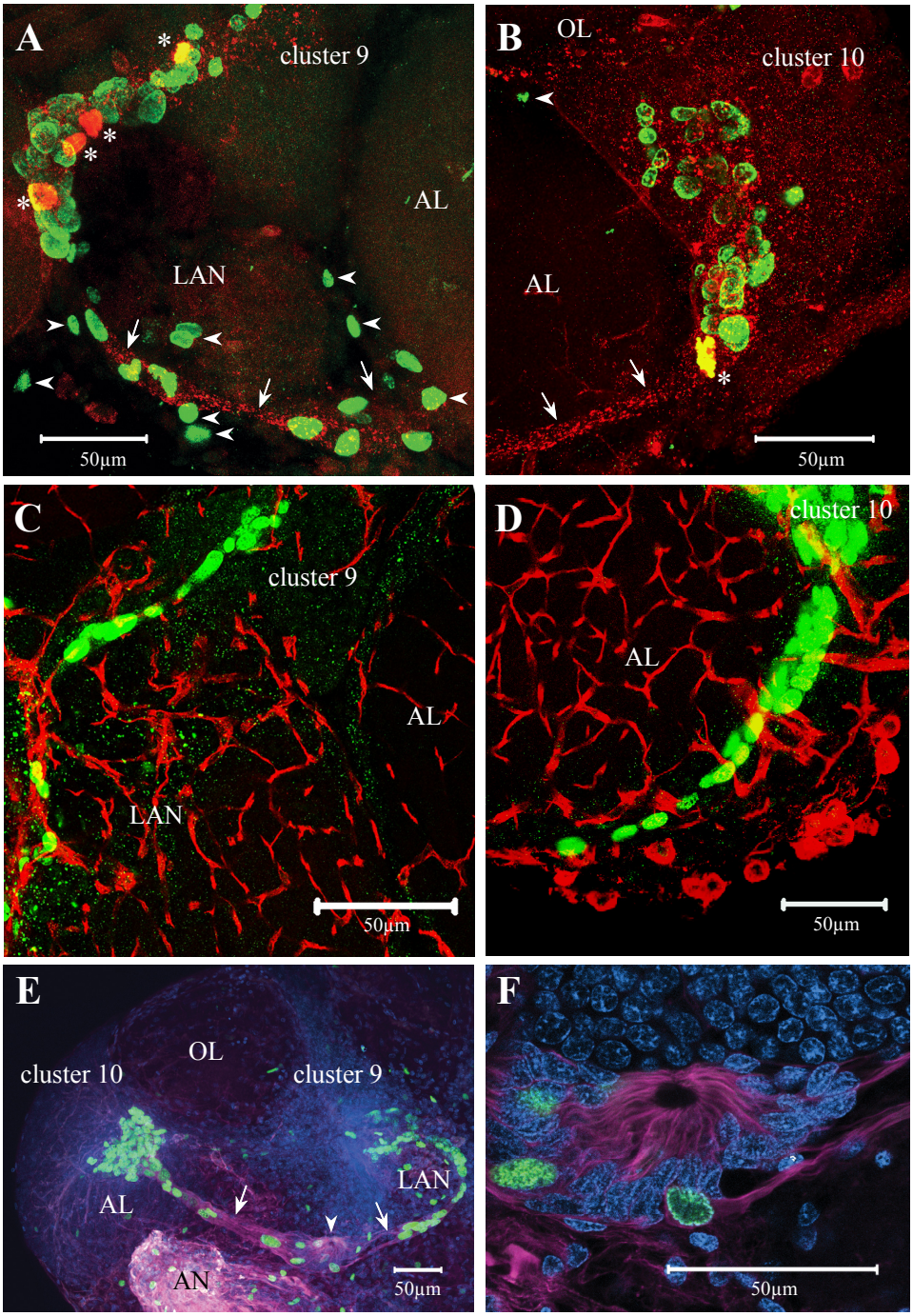

A
cluster 9
AL
LAN
50µm
B
OL
cluster 10
AL
50µm
C
cluster 9
AL
LAN
50µm
D
cluster 10
AL
50µm
E
OL
cluster 10
cluster 9
LAN
AL
AN
50µm
F
50µm

Fig. 10 Tubulin as an intracellular microtubule marker. Numerous cells are labeled with BrdU in the comma-shaped proliferation zone in the brain of crayfish hatchlings. **A.** A single confocal image showing BrdU+ cells in the comma-shape proliferation zone and one mitotic cell in cluster 10 (see arrow). Scale bar = 50 μm. **B.** A single confocal image showing the strand structure and microtubule aligned along the condensed DNA during mitosis (see arrow). Scale bar = 50 μm. **C.** A single confocal image showing nuclear staining. Scale bar = 50 μm. **D.** A Collapse of image in A-C. Scale bar = 50 μm.

Tubulin as an Intracellular Microtubule Marker

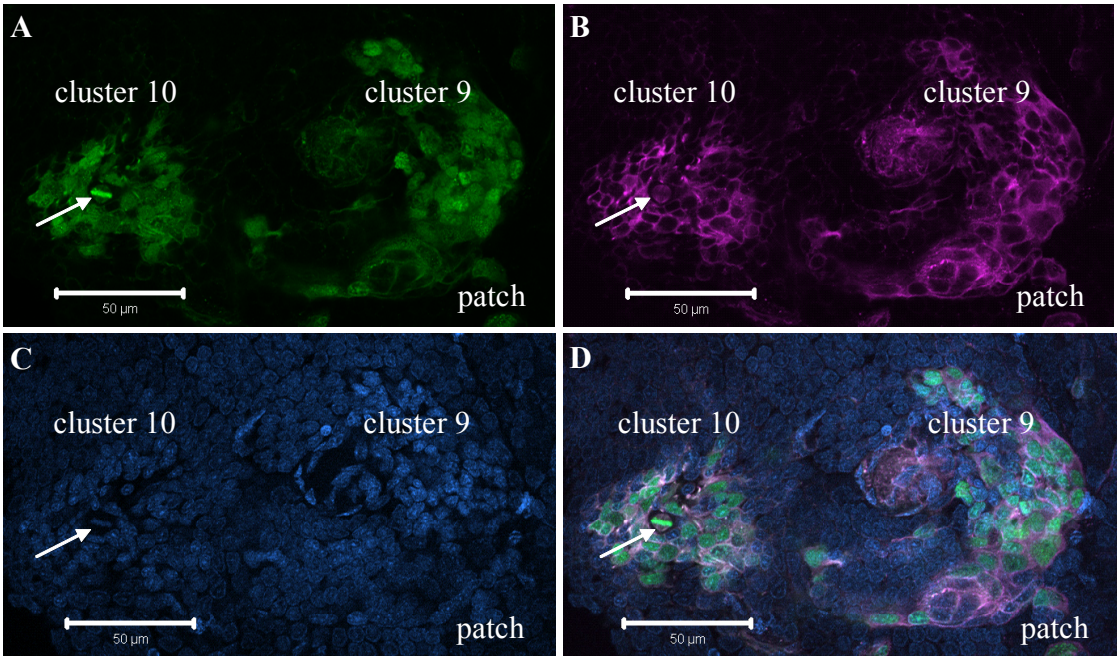


Fig. 11 Strand structure surrounding the comma-shaped proliferation zone in cluster 9 of fully grown adult crayfish. **A-C.** Stacks of confocal images taken at 0.5 μm interval. **A.** A few BrdU+ cells (green indicated by arrows) are present in the tail part of cluster 9. A patch of cells is present (see dotted circle). Scale bar = 100 μm. **B.** The strand structure that is positive to anti-H3P (red, arrowheads) is present in the tail part of cluster 9. Scale bar = 100 μm. **C.** Collapse of images in A and B shows that a few BrdU+ cells in the tail part of cluster 9 are surrounded by the strand structure. Scale bar = 100 μm.

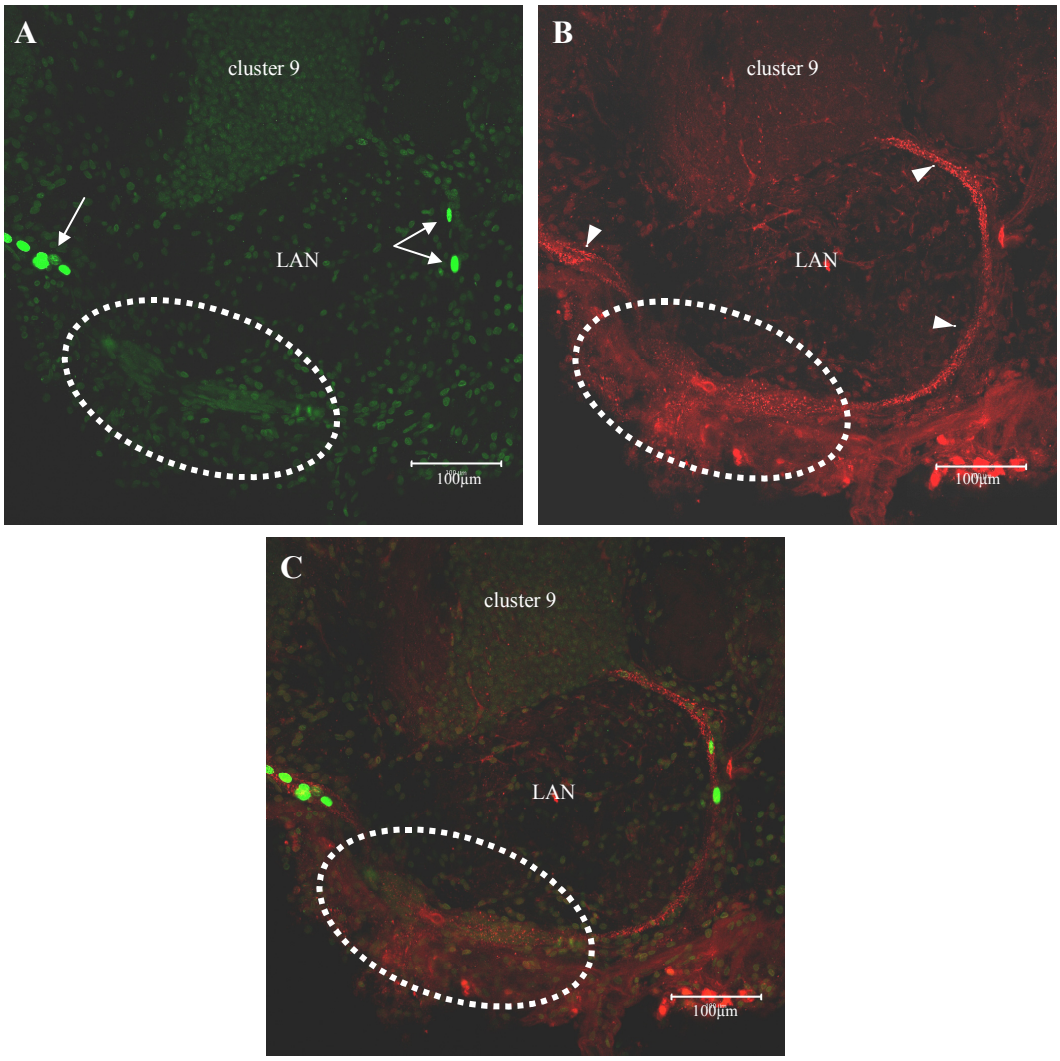
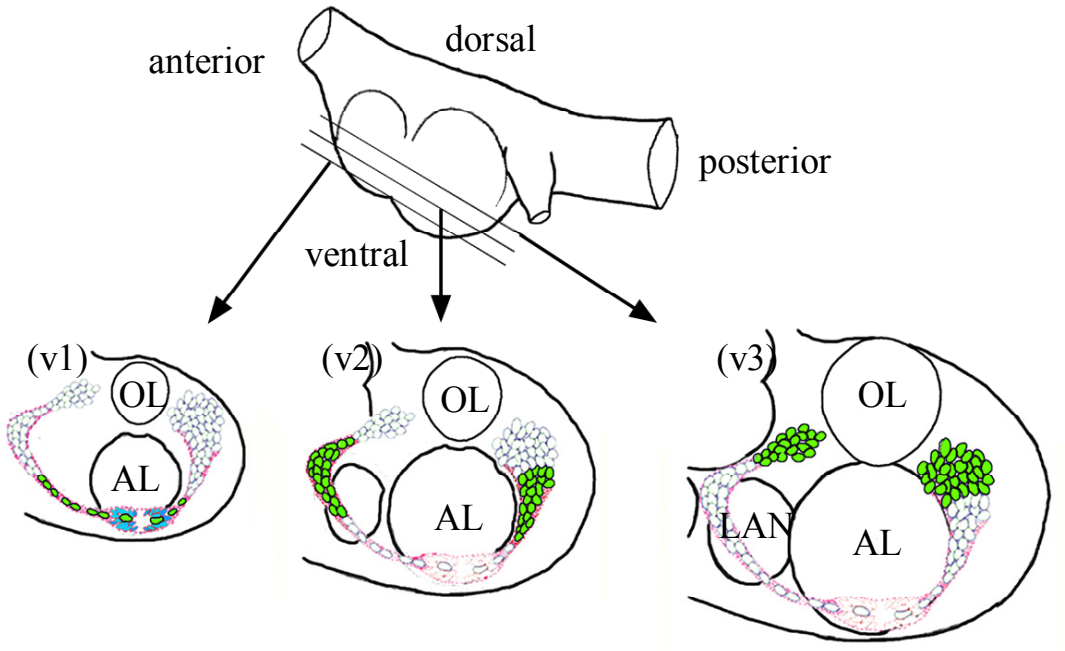

A
cluster 9
LAN
100µm
B
cluster 9
LAN
100µm
C
cluster 9
LAN
100µm

Fig. 12 Diagram of the comma-shape proliferation zone. **v1.** Near ventral surface of the brain, there are the patch of cells of lenticular shape (blue), and a line of BrdU+ cells (green) in the cluster 9 and 10 tail parts surrounded by the strand structure (pink). **v2.** Approximately 100 μm dorsal from v1, the tail parts of the comma for clusters 9 and 10 (green) surrounded by strand structure (pink) curve around the LAN (cluster 9) or around the lateral AL (cluster 10). **v3.** Approximately 100-300 μm dorsal from v2, the head parts of the comma for clusters 9 and 10 appear. A ball of BrdU+ cells (green) emerges within the cluster boundaries adjacent to the OL and the AL.


anterior
dorsal
posterior
ventral
(v1)
OL
AL
(v2)
OL
AL
(v3)
OL
LAN
AL

III.1.4. Distribution of Newborn Cells in the Comma-Shape Proliferation Zone

To further characterize the comma-shaped arrangement of the proliferation zone in crayfish brain, the distribution of newborn cells within the comma was examined with respect to their location, size and shape of their nuclei after three different pulse-chase time points: 1, 7, 14 days after 24 hr BrdU immersion (BrdU pulse). For these morphological analyses, all counted BrdU+ cells were divided into five different zones: the cluster 9 head part, the cluster 9 tail part, patch, cluster 10 tail part, and cluster 10 head part.

III.1.4.1. More Surviving Newborn Cells in the Head Part of the Comma

In the 1-day pulse-chase group, BrdU+ cells were evenly distributed in the cluster 9 head part (~50%) and in the cluster 9 tail part (~50%) (1d Pulse-Chase in Table 1). This is in contrast to the uneven distribution of newborn cells in the cluster 10 head part (~90%) and in the cluster 10 tail part (~10%) (1d Pulse-Chase in Table 1). After 7 days of survival time, however, the number of BrdU+ cells in the cluster 9 head part greatly increased to more than four times, whereas the number of BrdU+ cells in the cluster 9 tail part decreased to near half compared to the 1-day pulse-chase group (7d Pulse-Chase in Table 1). The distribution of newborn cells in the cluster 10 head part was relatively similar in the 1-day and the 7-day pulse-chase groups (7d Pulse-Chase in Table 1). However, the number of BrdU+ cells in the cluster 10 tail part decreased to half after 7 days survival time (7d Pulse-Chase in Table 1). The uneven distribution of BrdU+ cells

in cluster 9 manifested after 14 days of survival time. The number of BrdU+ cells was approximately nine times more in the cluster 9 head part compared to the number in the cluster 9 tail part (14d Pulse-Chase in Table 1). The uneven distribution was even more drastic for cluster 10 in the 14-day pulse-chase group (14d Pulse-Chase in Table 1). Only about 7 cells were present in the cluster 10 tail part, while more than 800 cells were present in the cluster 10 head part after 14 days of survival time (14d Pulse-Chase in Table 1). A number of BrdU+ cells within the patch of cells in the 1-day pulse-chase group doubled after 7 days of survival time (1d and 7d Pulse-Chases in Table 1). After 14 days of survival time, the number of BrdU+ cells greatly increased in the patch of cells (14d Pulse-Chase in Table 1). These results suggest that cells in the comma-shape proliferation zone are dividing.

The number of mitotic cells was similar in the cluster 9 head part and in the cluster 9 tail part in all pulse-chase test groups (Table 2). However, significantly more cells were going through mitosis in the cluster 10 head part compared to the cluster 10 tail part (Table 2). Due to high variance in the number of BrdU+ cells in the patch, the median value of mitotic cells in the patch was zero (Table 2). A few animals had mitotic cells in the patch, resulting in the maximum value of H3P+ cells to be four (Table 2).

Table 1. Number of BrdU+ Cells in Comma-Shape Neurogenic Areas

(minimum, 25% interquartile, **median**, 75% interquartile, maximum)

	Cluster 9 Head	Cluster 9 Tail	Cell Patch	Cluster 10 Tail	Cluster 10 Head
1-day Pulse-Chase (n = 44)	25 55 **82** 121 230	23 58 **91** 116 186	0 0 **2** 8 27	0 7 **16** 31 63	261 365 **401** 436 566
7-day Pulse-Chase (n = 17)	210 307 **331** 369 473	0 28 **57** 73 115	0 0 **4** 16 57	0 2 **8** 15 67	259 308 **357** 518 624
14-day Pulse-Chase (n = 18)	299 574 **719** 951 1111	0 44 **80** 122 178	0 0 **25** 54 106	0 0 **7** 21 33	457 595 **887** 1156 1479

Table 2. Number of H3P+ Cells in Comma-Shape Neurogenic Areas

(minimum, 25% interquartile, **median**, 75% interquartile, maximum)

	Cluster 9 Head	Cluster 9 Tail	Cell Patch	Cluster 10 Tail	Cluster 10 Head
1-day Pulse-Chase (n = 44)	0 0 **2** 4 8	0 0 **2** 3 6	0 0 **0** 0 1	0 0 **0** 0 4	0 7 **9** 12 28
7-day Pulse-Chase (n = 17)	0 1 **2** 3 5	0 0 **0** 1 3	0 0 **0** 0 4	0 0 **0** 0 4	0 1 **2** 3 4
14-day Pulse-Chase (n = 18)	0 1 **3** 6 9	0 1 **3** 7 10	0 0 **0** 0 4	0 0 **0** 1 4	7 9 **11** 15 18

III.1.4.2. More Newborn Cells of Smaller Nuclei in the Head Part of the Comma

To determine if the head part of the comma-shaped proliferation zone has different characteristics from the tail part, semi-thin brain sections were stained with methylene blue. The ventral regions of the brain were examined and the tail part of the comma shape neurogenic area in cluster 9, as shown in diagrams in Fig. 12, was identified. Methylene blue staining of ventral planes of cluster 9 indicated that the cells in the tail part, which would be normally labeled with BrdU around the LAN, possessed large nuclei with elongated cytoplasm (Fig. 13A; red circles in the cluster 9 tail part), while the cells in the head part possessed small nuclei with thin cytoplasm (Fig. 13A; pink circles in the cluster 9 head part). A patch of cells, that would normally have a few BrdU+ cells, had a center devoid of cells and around which cells of lenticular shape clustered (Fig. 13B).

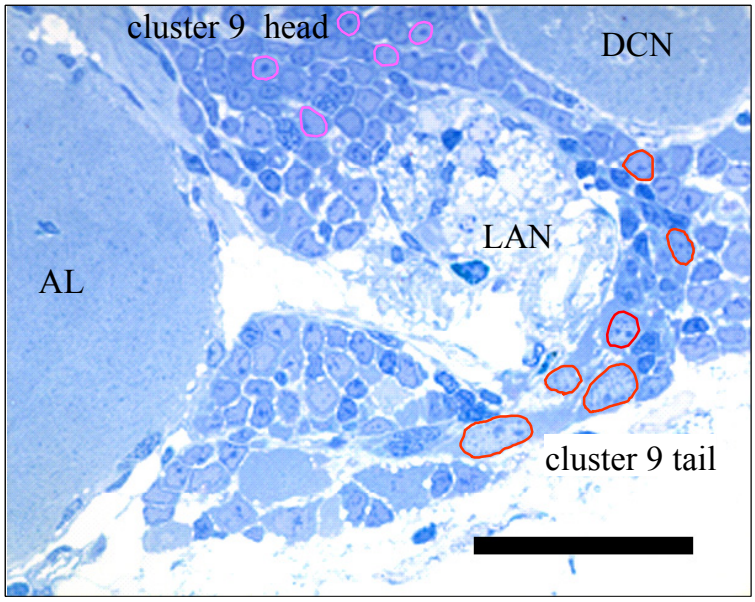
More than 30,000 BrdU+ cells were examined in cell cluster 9 and more than 42,000 BrdU+ cells examined in cell cluster 10. The size of the individual BrdU+ nuclei in the head part mostly ranged from 5-10 μm in diameter in all pulse-chase groups (Fig. 14A). The number of BrdU+ nuclei of 7-8 μm greatly increased in the cluster 9 head part after 7 days and 14 days of survival time (Fig. 14A). A similar size distribution of BrdU+ nuclei was observed in the cluster 10 head part, with increasing number of BrdU+ nuclei around 7-8 μm after 14 days of survival time (Fig. 14B). On the other hand, the tail part of the comma for both clusters 9 and 10 contained BrdU+ nuclei of large diameter, although their numbers are very low, compared to the head part in all pulse-chase groups (Fig. 14C-14D). In the cluster 9 tail part, there were most BrdU+ nuclei of

8-9 μm in the 1-day and the 7-day pulse-chase groups, and the size distribution of BrdU+ nuclei was skewed toward 7-8 μm after 14 days of survival time (Fig. 14C). In the cluster 10 tail part, BrdU+ nuclei ranged from 7-13 μm in diameter in all pulse-chase groups and only a few BrdU+ nuclei ranged between 7-8 μm (Fig. 14D). This result suggests that most BrdU+ cells in the cluster 10 tail part possessed larger nuclei than BrdU+ cells in the cluster 10 head part (Fig. 14D). A patch of cells in the 14-day pulse-chase group also contained large BrdU+ nuclei, although their number was low, compared to the head part of the comma (Fig. 14E).

Fig. 13 Methylene blue staining of juvenile crayfish brain. A. Cluster 9 comma-shaped proliferation zone. Cells that would be normally labeled with BrdU around the LAN, the tail part of cluster 9, have big nuclei (big circles in red) with elongated cytoplasm. Cells in the cluster 9 head part, on the other hand, have small nuclei (small circles in red) with thin cytoplasm. B. A patch, that would normally have a few BrdU+ nuclei, had a center devoid of cells. These images were kindly provided by Ms. Laurel M. Johnstone.

Methylene Blue Staining of Comma-Shaped Proliferation Zone in Cluster 9

A. Cluster 9 comma-shaped proliferation zone



B. A patch of cells

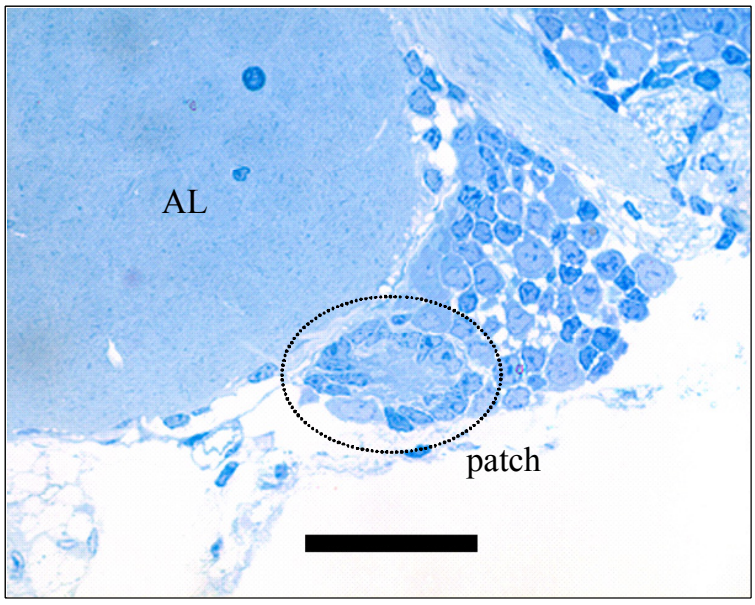
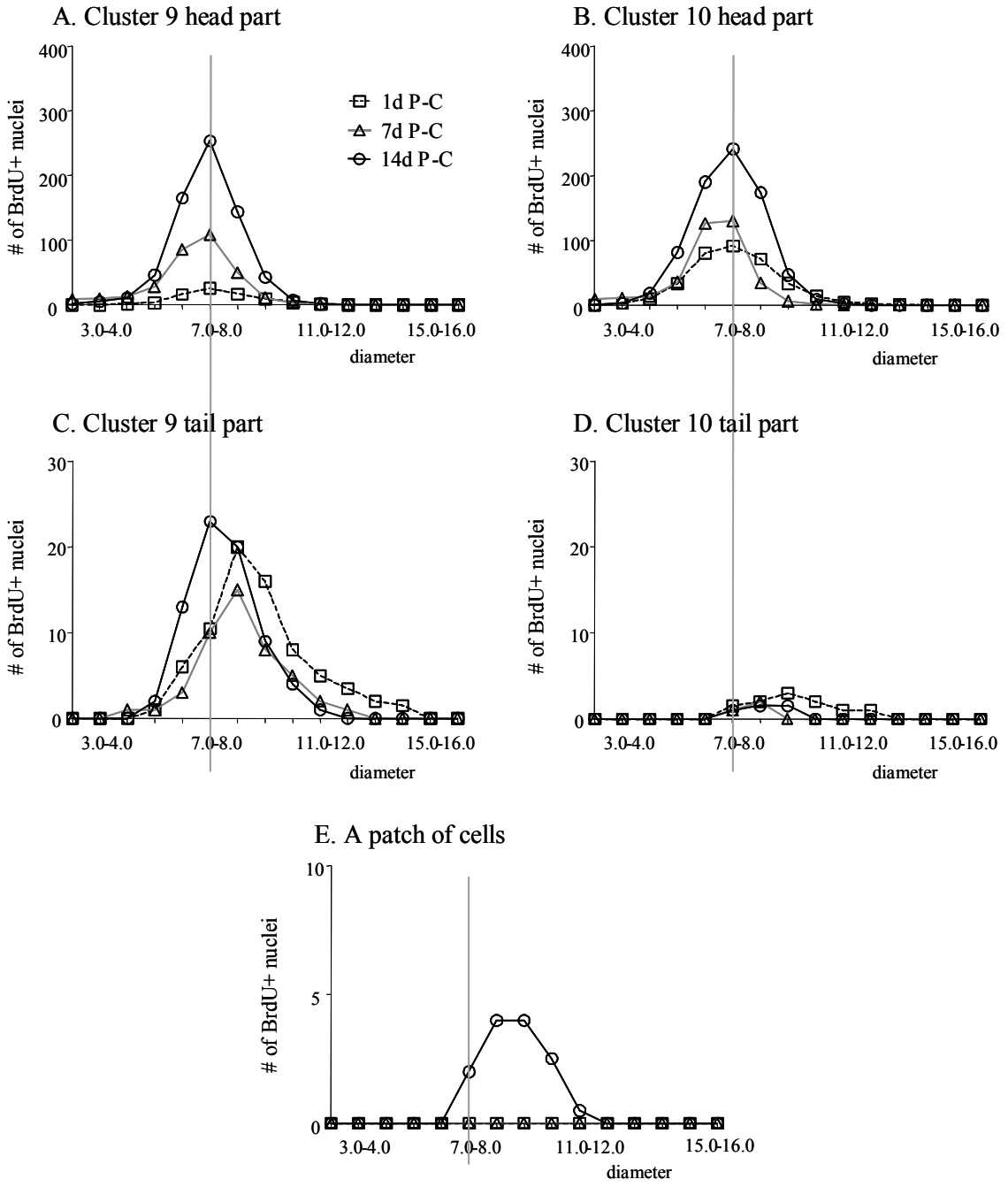


Fig. 14 Size of BrdU+ nuclei in comma-shaped proliferation zones. Values are shown in median. The gray lines represent the diameter range, 7-8 μm, where BrdU+ nuclei in the head parts of proliferation zone were mostly distributed. **A-B.** Clusters 9 and 10 head parts. The number of BrdU+ nuclei of 7-8 μm was low in 1 day pulse-chase group, and it increased after 7 and 14 days of survival times. **C.** Cluster 9 tail part. Most BrdU+ nuclei in 1 day and 7 day pulse-chase groups had diameter around 8-9 μm. After 14 days of survival time, most BrdU+ nuclei had 7-8 μm diameter range. **D.** Cluster 10 tail part. The number of BrdU+ nuclei was lower compared to cluster 9 tail part. BrdU+ nuclei in 1 day pulse-chase group had greater size, ranged up to 12.0 μm. Only a few BrdU+ nuclei in cluster 10 tail part had 7-8 μm diameter range. **E.** A patch of cells. Only a few BrdU+ nuclei were present in the patch of cells, and BrdU+ nuclei in the patch in 14 day pulse-chase group had large diameter range, 6-13 μm compared to those in the head parts. [an open circle stands for 1 day pulse-chase group (n = 44), an open triangle for 7 day pulse-chase group (n = 17), an open square for 14 day pulse-chase group (n = 18); note that the scales of Y axis were adjusted for clear data presentation]

Size of BrdU+ nuclei in Clusters 9 and 10 (median)



III.1.4.3. More Newborn Cells with Round Nuclei in the Head Part of the Comma

In the cluster 9 head part, the number of BrdU+ nuclei of R = 0.8-0.85 (R = 1.0 being a perfect circle, see Methods) increased after 7 days and 14 days of survival time. The center of the distribution of BrdU+ nuclei was not apparent in the 1-day pulse-chase group, however, it became apparent at 0.8-0.85 in the 7-day and the 14-day pulse-chase groups (Fig. 15A). For the cluster 10 head part, there were more BrdU+ nuclei with irregular shape (R = 0.75-0.8) in the 1-day pulse-chase group (Fig. 15B). After 7 days of survival time, however, the centered distribution of BrdU+ nuclei moved to 0.8-0.85, suggesting that the surviving newborn cells had BrdU+ nuclei with a rounder shape than those of the 1-day pulse-chase group (Fig. 15B). After 14 days of survival time, the number of BrdU+ nuclei of R = 0.8-0.85 approximately doubled (Fig. 15B). A similar shape distribution of BrdU+ nuclei and a shift of the peak distribution also occurred in the cluster 9 tail part (Fig. 15C). In the cluster 10 tail part, only a small number of BrdU+ nuclei had R = 0.8-0.85 and most BrdU+ nuclei in the 1-day and the 14-day pulse-chase groups had lower R values, suggesting that the BrdU+ nuclei in the cluster 10 tail part had an irregular, i.e., more elongated, shape compared to those in the head part (Fig. 15D). A bimodal distribution was observed among a small number of BrdU+ nuclei in a patch of cells of 14 days pulse-chase group: one population of BrdU+ nuclei displayed a peak distribution at R = 0.7-0.75, while the other population of BrdU+ nuclei displayed a peak distribution at R = 0.8-0.85 (Fig. 15E).

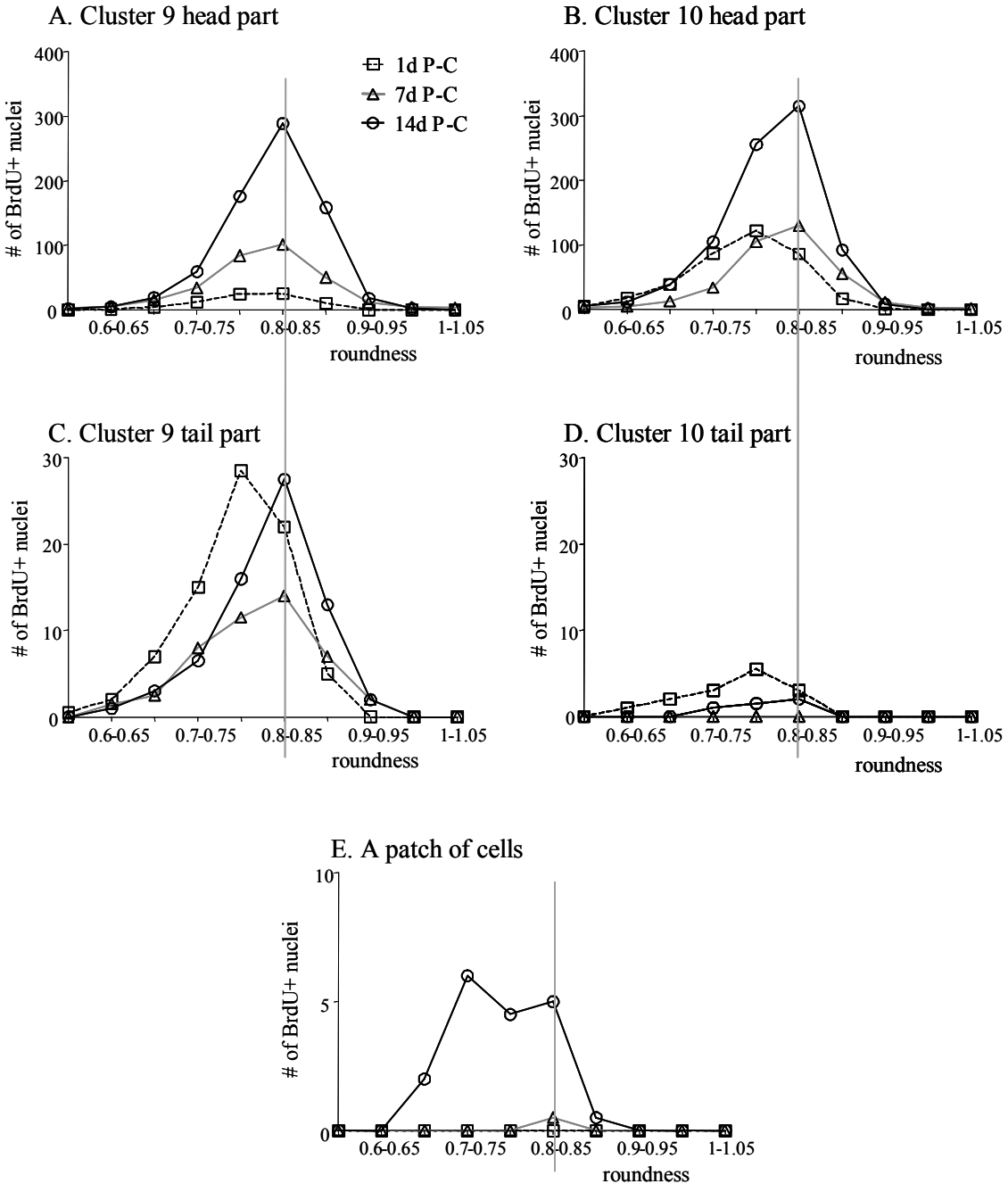
The roundness of the individual BrdU+ nuclei in the cluster 9 head and tail parts of the 1-day pulse-chase group was R=0.78±0.06 (mean±SD); and that of the 7-day

pulse-chase group was R=0.80±0.08 (mean±SD); and that of the 14-day pulse-chase groups was R=0.81±0.06 (mean±SD). The roundness of the individual BrdU+ cells in the cluster 10 head and tail parts in 1 day pulse-chase group was R=0.76±0.07 (mean±SD); and that of the 7-day pulse-chase group was R=0.80±0.08 (mean±SD); and that of the 14-day pulse-chase groups was R=0.79±0.06 (mean±SD).

Other studies had shown that most cells in clusters 9 and 10 are mature neurons by having synaptic contacts and neurotransmitter expression (Schmidt, 2001; Sullivan and Beltz, 2005). Therefore, the size and shape of unlabeled cells in clusters 9 and 10 were also measured assuming that they are mature neurons and neither glia nor blood cells (Harzsch et al., 1999; Schmidt, 2001). The size of the nuclei of unlabeled mature neurons in the cluster 9 head part ranged from 5-12 μm in diameter with a peak distribution at 7-8 μm (D=7.92±1.24, mean±SD), with a more regular shaped nuclei that was closer to a circle (R=1.00±0.02, mean±SD) in all pulse-chase groups (150 nuclei from 15 brains). The size of the nuclei of unlabeled mature neurons in the cluster 10 head part ranged from 6-11 μm in diameter with a peak distribution at 8-9 μm (D=8.45±0.85, mean±SD), with a more regular shaped nuclei that was closer to a perfect circle (R=1.00±0.01, mean±SD) in all pulse-chase groups (150 nuclei from 15 brains).

Fig. 15 Shape of BrdU+ nuclei in comma-shaped proliferation zones. Values are shown in median. The gray lines represent the roundness range, R = 0.8-0.85 (when R of a perfect circle is 1.0), where most BrdU+ nuclei in the head parts of proliferation zone distributed. **A.** Cluster 9 head part. The number of BrdU+ nuclei of R = 0.8-0.85 was low in 1 day pulse-chase group, and it increased after 7 and 14 days of survival times. **B.** Cluster 10 head part. Most BrdU+ nuclei in 1 day pulse-chase group ranged around R = 0.75-0.8. After 7 and 14 days of survival time, most BrdU+ nuclei distributed around R = 0.8-0.85. **C.** Cluster 9 tail part. Most BrdU+ nuclei in 1 day pulse-chase group ranged around R = 0.75-0.8. After 7 and 14 days of survival time, most BrdU+ nuclei distributed around R = 0.8-0.85. **D.** Cluster 10 tail part. The number of BrdU+ nuclei was lower compared to cluster 9 tail part. BrdU+ nuclei in 1 day and 14 days pulse-chase groups had smaller R value. Only a few BrdU+ nuclei in cluster 10 tail part had R = 0.8-0.85. **E.** A patch of cells. Only a few BrdU+ nuclei were present in the patch of cells. Some population of BrdU+ nuclei in the patch in 14 day pulse-chase group had smaller R value, 0.7-0.75, while other population of BrdU+ nuclei had greater R value, 0.8-0.85. [an open circle stands for 1 day pulse-chase group (n = 44), an open triangle for 7 day pulse-chase group (n = 17), an open square for 14 day pulse-chase group (n = 18); note that the scales of Y axis were adjusted for clear data presentation]

Shape of BrdU+ nuclei in Clusters 9 and 10 (median)



III.2. Social Status-Dependent Changes in Cell Proliferation and Survival

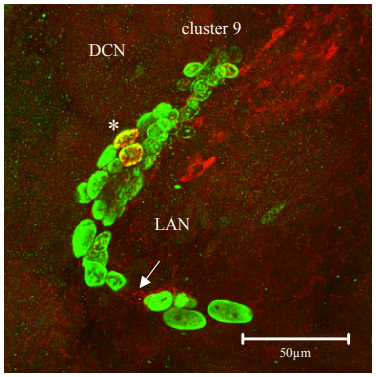
To examine the effect of social status on juvenile neurogenesis in the crayfish brain, the counts of proliferating and surviving BrdU+ cells as well as the counts of H3P+ cells in the whole comma shape proliferation zone were compared in isolate, dominant, and subordinate animals. In all cell proliferation and survival test groups, proliferating and surviving newborn cells appeared within a comma-shaped proliferation zone both in clusters 9 and 10 irrespective of the social status of animals (Fig. 16). As described in the section above, the number of BrdU+ cells differed between the head and tail regions of the comma, typically with significantly more cells in the head than in the tail. The effects of social experience on cell number were generally similar for the head and tail regions; thus in the figures and tables representing these data, I use the counts from the entire comma (i.e., the sum of the head and tail) and describe in the text any differences in effects of social experience on cell counts in head vs. tail regions. This is also true for the H3P+ cells.

Fig. 16 'Comma-shaped' cell proliferation and cell survival. Proliferating and surviving cells are present in cell clusters 9 (**A-C**) and 10 (**D-F**) after 1 day, 7 days, and 14 days of social interaction. BrdU+ cells are shown in green and H3P+ cells are shown in yellow or in orange depending on the amount of BrdU/H3P colocalization (asterisks). Arrows indicate strand structure surrounding BrdU+ cells. Counted BrdU+ cells are marked with white lines in (**B-C**) and (**E-F**) for clarity. Stacks of confocal images taken at 0.5μm interval. Scale bar = 50 μm in all images. **A-B.** Lines of BrdU+ cells are present in the ventral posterior part of cell cluster 9 and around the lateral antennular neuropil (LAN) after 1 day (**A**) and 7 days (**B**) of social interaction. BrdU+ cells were present in the same location in all cell proliferation tests irrespective of the length of social interaction. **C.** After 14 days of survival time, BrdU+ cells were faintly labeled around the anterior edge of the LAN where several H3P+ cells were observed. **D-F.** A ball of BrdU+ cells appeared in cell cluster 10 near the olfactory lobe (OL) and accessory lobe (AL) after 1 day (**D**), 7 days (**E**), and 14 days (**F**) of social interaction. Regardless of the length of social interaction, all cell proliferation tests produced a ball of BrdU+ cells in the same location of cell cluster 10. As the survival time increased, the center of the ball of BrdU+ cells was fainter than the outer ring of BrdU+ cells, indicating the dilution of BrdU due to cell division. AL, accessory lobe; DCN, deutocerebral commissure neuropil; LAN, lateral antennular neuropil; OL, olfactory lobe.

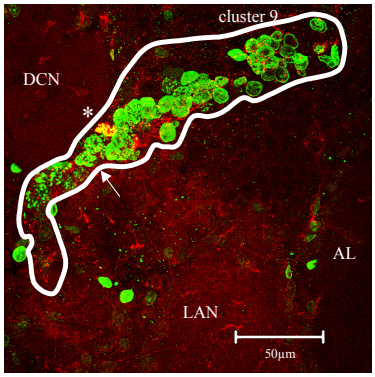
Comma-Shaped Proliferation Zone: Proliferation and Survival of Newborn Cell

Cluster 9

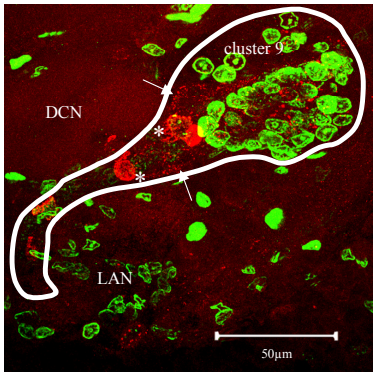
A. 1 day cell proliferation



B. 7 days cell survival

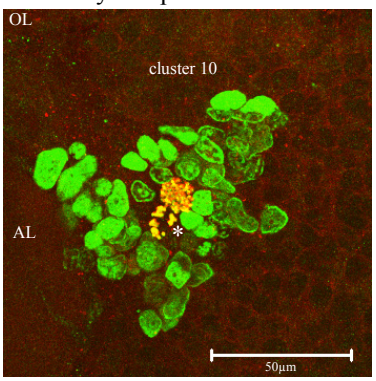


C. 14 days cell survival

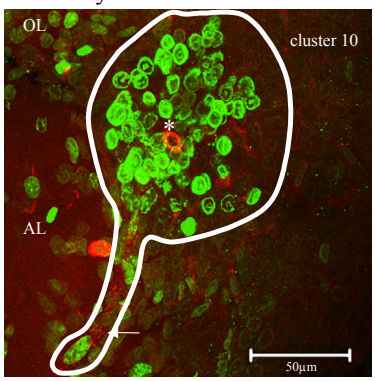


Cluster 10

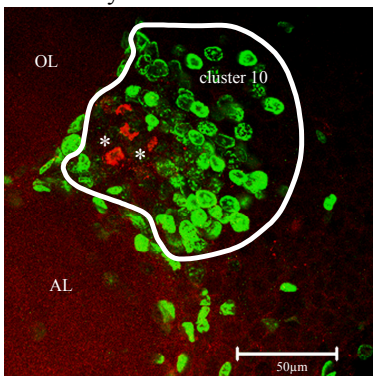
D. 1 day cell proliferation



E. 7 days cell survival



F. 14 days cell survival



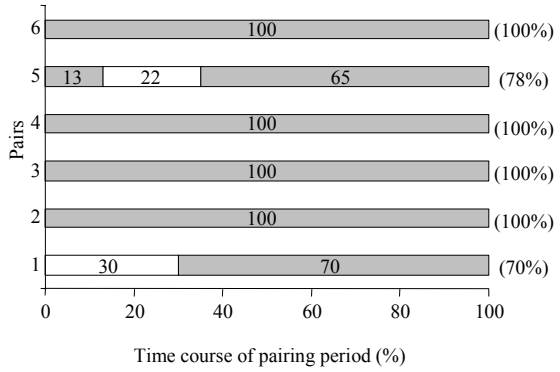
BrdU BrdU≈H3P BrdU<H3P H3P

III.2.1. Degree of Domination

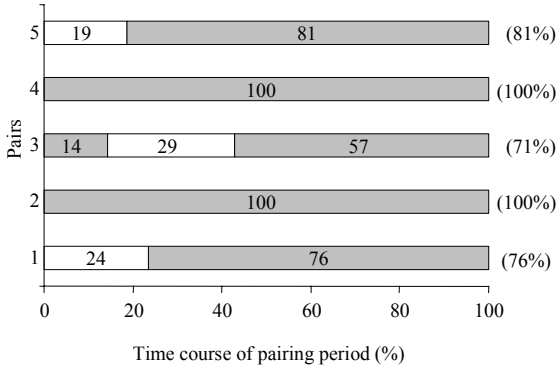
Behavioral observations were performed each day in the morning to identify the social status (dominant or subordinate) of each animal. Each observation was up to 5 min for each pair of animals. 'Dominant' status was assigned to an animal when it solely occupied the shelter or approached, chased, or attacked its opponent, causing the opponent to retreat. The opponent was designated as the 'subordinate'. Subordinate animals often clung to the mesh wall, stayed at the rim of the arena, or retreated or tailflipped away from their dominant opponents. A stable dominance hierarchy was considered to be established when one animal stayed dominant over its partner, either by occupying the shelter alone or by displaying aggressive behaviors, for more than 70% of the total pairing period. In the 1 day cell proliferation test group, five of 10 pairs formed and maintained 100% stable dominance hierarchies and had two intact antennular lateral flagella, and thus were included in analysis. Thus the degree of dominance of these five pairs was not shown in Fig. 17. The other five pairs in the 1 day cell proliferation test group were excluded because two pairs experienced status reversal overnight, one pair did not form a clear dominance hierarchy overnight, and two pairs had damaged antennular lateral flagella. Out of 28 pairs examined from 7 and 14 days experiments, 20 pairs maintained a 100% stable dominance hierarchy, while 8 pairs experienced brief periods of status reversal (Fig. 17).

Fig. 17 Degree of dominance within pairs of animals in the 7- and 14-day tests. Stacked bar represents the total interaction period in each test. Numbers within white / gray bars represent time period that one member of the pair was dominant over its partner. Proportion values in parentheses represent the total time period that the eventual dominant animals maintained dominance status. **A.** Among six pairs used in 7 day cell proliferation test, only two pairs experienced status reversal, and these reversals did not correlate with molting. **B.** Among five pairs used in 14 day cell proliferation test, three pairs experienced status reversal during their interaction period. Neither member of pairs 1 and 5 molted. The subordinate animal in pair 3 acquired dominance status briefly before and after the molting of the dominant animal. **C.** Among six pairs used in 7 day cell survival test, only one pair experienced brief reversal of dominance status at early phase of interaction period. Neither member of this pair molted during the 7 days. **D.** Animals used in 14 day cell survival test. Only two pairs experienced status reversal during their interaction period. This was not due to molting in the case of pair 1, but the brief status reversals in pair 4 were preceded or followed by molting of the subordinate animal.

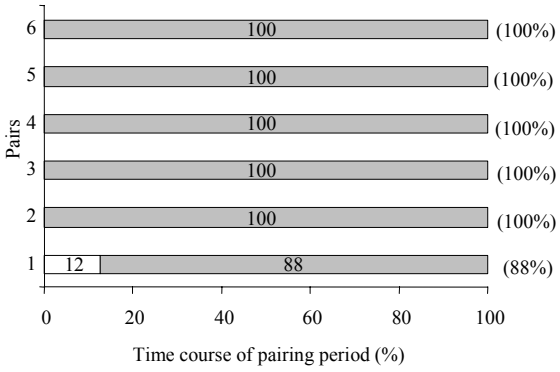
A. Pairs in 7 day Proliferation Test



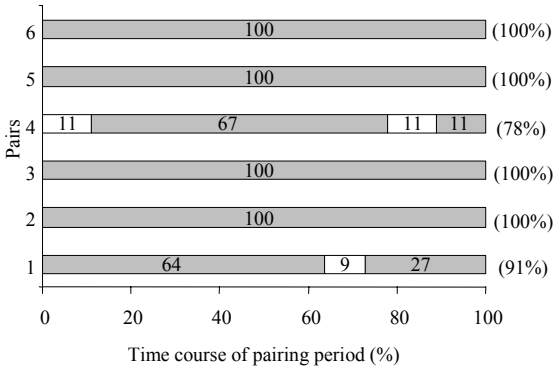
B. Pairs in 14 day Proliferation Test



C. Pairs in 7 day Survival Test



D. Pairs in 14 day Survival Test



III.2.2. Damage to Antennular Lateral Flagella

In many crustaceans including crayfish, the antennular lateral flagellum, the chemosensory organ, is vulnerable to damage during social interactions. A complete loss of one lateral flagellum has been shown to reduce the level of neurogenesis in adult shore crabs (Hansen and Schmidt, 2001). Thus, the best efforts were made to obtain as many dominant-subordinate pairs as possible having intact lateral flagella throughout 14 days of social interaction. Despite these efforts, some animals had damage – loss of up to 70% – of one lateral flagellum. As expected, subordinate animals that had 14 days of social interaction suffered the most damage. For the 14 days cell proliferation test, only one subordinate animal out of 10 pairs managed to retain two fully intact lateral flagella after the experience of a stable dominance hierarchy. For the 14 days cell survival test, six subordinate animals out of 20 pairs retained fully intact lateral flagella after the experience of a stable dominance hierarchy. Unlike in Hansen and Schmidt's (2001) study of adult shore crabs, the number of BrdU+ cells in the side of brain ipsilateral to the damaged lateral flagellum was not greatly reduced compared to the side with the intact lateral flagellum in the juvenile crayfish examined in this study. This difference may be due to, the differing amounts of damage (complete loss vs. partial damage). The numbers of BrdU+ cells in the brains of animals with intact lateral flagella and those with partial damage to one lateral flagellum are listed for comparison (Table 3).

III.2.3. Cell Proliferation Tests for Cell Cluster 9

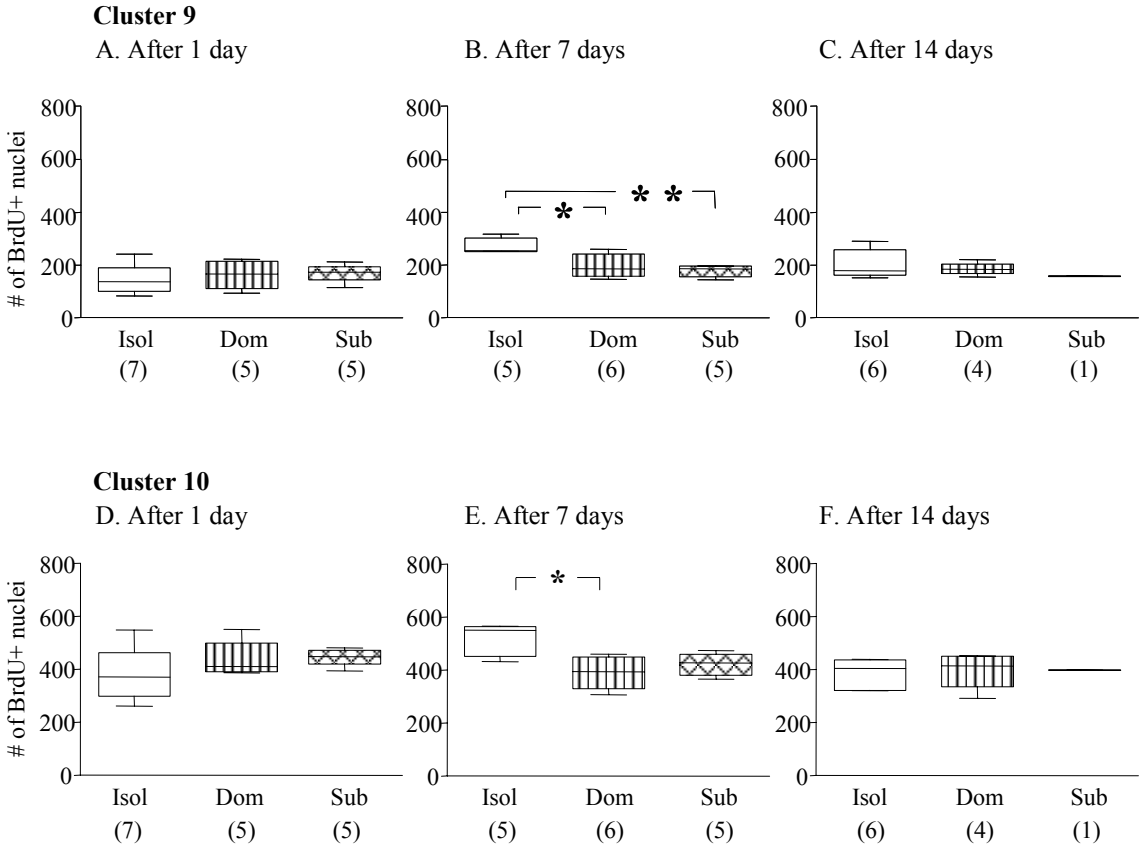
In cell proliferation tests for cell cluster 9 (Fig. 18A-18C, Table 3), the number of BrdU+ cells was not different between isolates, dominants, and subordinates after 1 day of social experience (Fig. 18A; overall difference $p = 0.872$). No difference was found in the number of BrdU+ cells of dominants vs. subordinates after 7 days of social experience (Fig. 18B; for 7 days: $p_{D \text{ vs } S} = 0.4848$). Unexpectedly, however, animals in isolation produced significantly more BrdU+ cells in cluster 9 than animals that experienced a dominance hierarchy after seven days of social interaction (Fig. 18B; overall difference $p = 0.0182$, $p_{I \text{ vs } D} = 0.030$, $p_{I \text{ vs } S} = 0.0079$). This increased cell proliferation in isolate animals was not present for 14 days interaction (Fig. 18C; overall difference $p = 0.6371$). On the other hand, the numbers of BrdU+ cells in dominants and subordinates stayed relatively constant throughout 1, 7, and 14 days of social interactions (Fig. 18A-18C). The effect of dominant-subordinate relationship on the number of BrdU+ cells after 14 days of social experience could not be compared because only one subordinate animal out of ten pairs retained two fully intact lateral flagella after experiencing a stable dominance hierarchy (Fig. 18C). Similar results were obtained when BrdU+ cells in the head and tail parts of the comma were counted separately.

III.2.4. Cell Proliferation Tests for Cell Cluster 10

In cell proliferation tests for cell cluster 10 (Fig. 18D-18F, Table 3), the number of BrdU+ cells was not different between isolates, dominants, and subordinates after 1 day of social experience (Fig. 18D; overall difference $p = 0.318$). Similar to the result from the cell proliferation test in cluster 9, the number of BrdU+ cells in cluster 10 of dominants and subordinates did not differ ($p_{D \text{ vs } S} = 0.4286$) after seven days of social interaction. However, animals in isolation produced transiently more BrdU+ cells in cluster 10 than did animals that experienced a dominance hierarchy for 7 days (Fig. 18E; overall difference $p = 0.0298$, $p_{I \text{ vs } D} = 0.0173$, $p_{I \text{ vs } S} = 0.0556$). After 14 days, the numbers of BrdU+ cells in cluster 10 were not different in all three groups of animals (Fig. 18F; overall difference $p = 0.9062$). Results for the cells in the head and tail regions of the comma-shaped group of BrdU+ cells were similar to those of the entire group.

Fig .18 Cell Proliferation Test for Cell Clusters 9 and 10. Number of BrdU+ cells in cluster 9 (**A-C**) and in cluster 10 (**D-F**) after 1 day, 7 days, and 14 days of social interaction. Values are the sum of labeled cells from the two bilateral cell cluster 9s of a brain, and expressed as median ± interquartile range. **A and D.** After 1 day of social interaction, the number of BrdU+ cells did not differ for dominant, subordinate, and isolate animals (Kruskal-Wallis test for overall difference: $p_{cl\ 9} = 0.872$; $p_{cl\ 10} = 0.318$). **B and E.** After 7 days of social interaction, isolate animals had significantly more cluster 9 BrdU+ cells than dominant and subordinate animals (Kruskal-Wallis test for overall difference: $p = 0.018$; Mann-Whitney test: $p_{I\ vs\ D} = 0.030$, $p_{I\ vs\ S} = 0.0079$; $p_{D\ vs\ S} = 0.662$). Similarly, after 7 days of social interaction, isolate animals had significantly more cluster 10 BrdU+ cells than dominant animals (Kruskal-Wallis test for overall difference: $p = 0.0298$; Mann-Whitney test: $p_{I\ vs\ D} = 0.0173$, $p_{I\ vs\ S} = 0.0556$; $p_{D\ vs\ S} = 0.4286$). **C and F.** After 14 days of social interaction, the number of BrdU+ cells did not differ for dominant, subordinate, and isolate animals (Kruskal-Wallis test for overall difference: $p_{cl\ 9} = 0.637$; $p_{cl\ 10} = 0.9062$). Further comparison was not performed with the subordinate because only one subordinate animal retained fully intact antennular lateral flagella after experiencing 14 days of stable dominance hierarchy. (I, isolate animals; D, dominant animals; S, subordinate animals; the numbers in parentheses indicate the number of animals examined).

Cell Proliferation Test (median ± range)



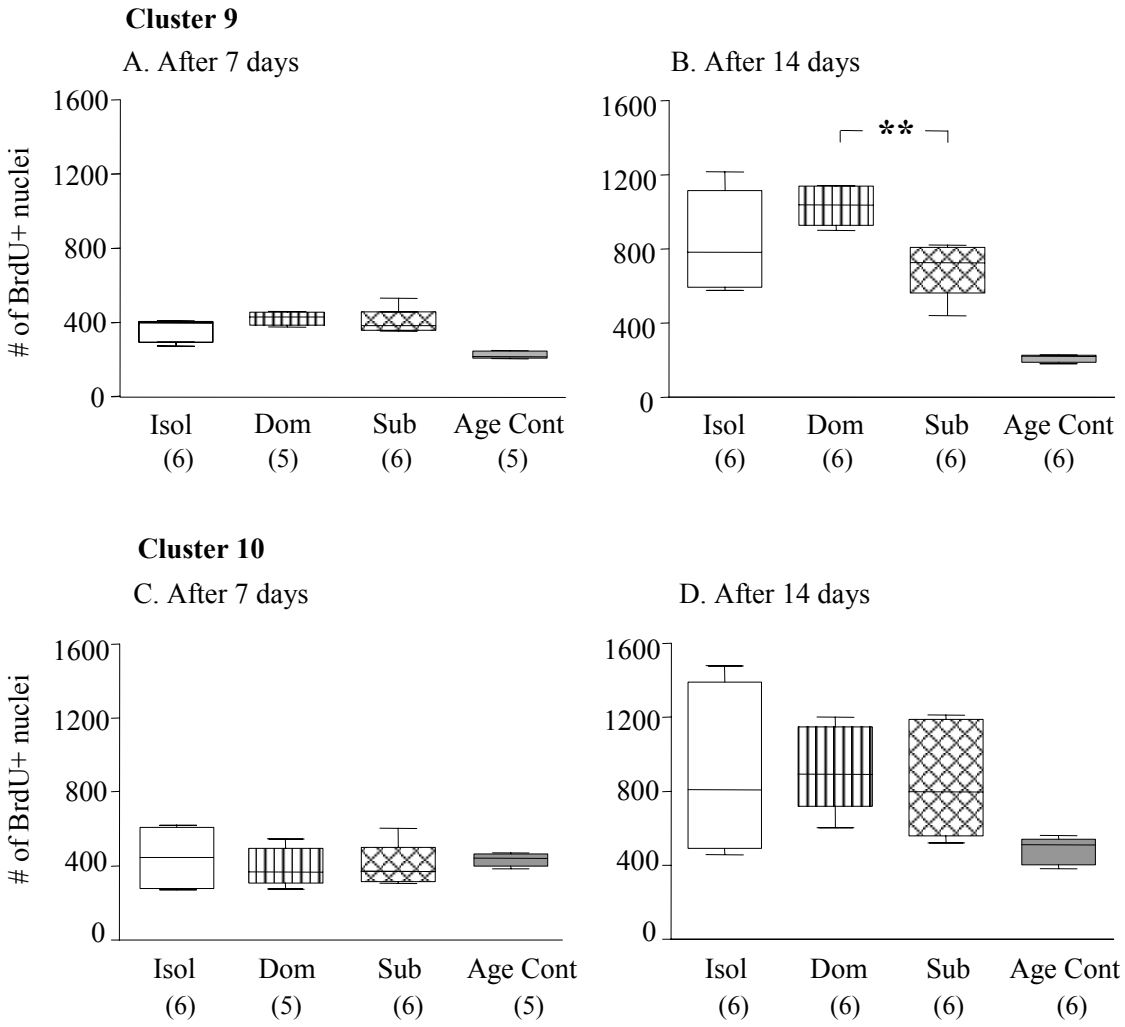
III.2.5. Cell Survival Tests for Cell Cluster 9

In cell survival tests for cell cluster 9 (Fig. 19A-19B, Table 3), the number of surviving BrdU+ cells after 7 days of social interaction was not influenced by the social status of an animal (Fig. 19A; overall difference $p = 0.2823$). However, after 14 days of social experience, dominant animals had significantly more surviving BrdU+ cells compared with subordinate animals (overall difference $p = 0.0294$, $p_{D \text{ vs } S} = 0.0022$) but not compared to animals isolated for 14 days ($p_{I \text{ vs } D} = 0.1797$, $p_{I \text{ vs } S} = 0.6991$) (Fig. 19B). This effect was similar for both the head and tail parts of the BrdU+ cell group.

III.2.6. Cell Survival Tests for Cell Cluster 10

In cell survival tests for cell cluster 10 (Fig. 19C-19D, Table 3), the numbers of BrdU+ cells in cluster 10 that survived after 7 days and 14 days of social interaction were not influenced by the social status of an animal (Fig. 19C; 7 day: $p = 0.9780$, Fig. 19D; 14 day: $p = 0.9268$). This effect was similar for the head and tail parts of the comma of BrdU+ cells.

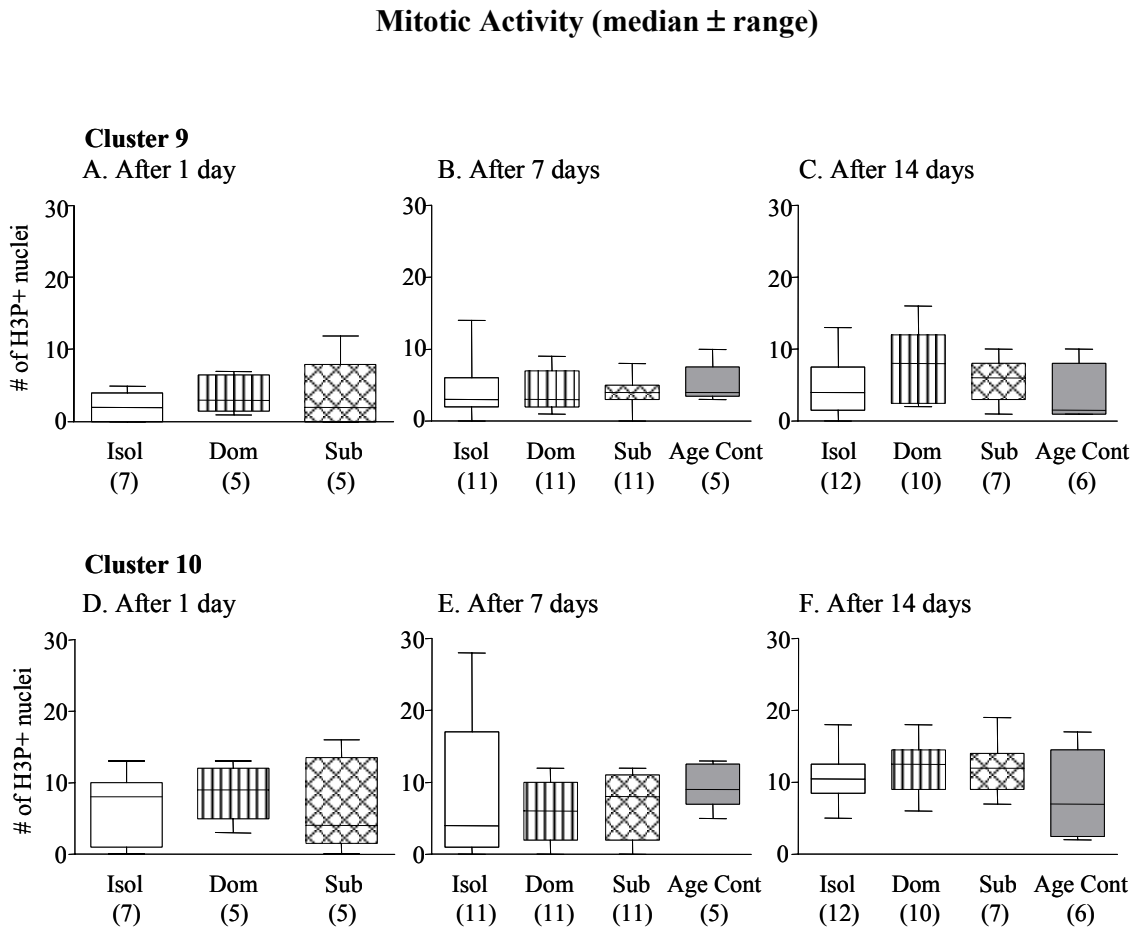
Fig. 19 Cell Survival Test for Cell Clusters 9 and 10. Number of BrdU+ cells in cluster 9 (**A-B**) and in cluster 10 (**C-D**) after 7 days and 14 days of social interaction. Values are the sum of labeled cells from the two bilateral cell cluster 9s of a brain, and expressed as median ± interquartile range. **A.** Seven days of social interaction did not affect the number of surviving cluster 9 BrdU+ cells (Kruskal-Wallis test for overall difference: $p = 0.2823$). The number of BrdU+ cells in the 3 experimental groups doubled relative to that of the age-control animals. **B.** After 14 days of social interaction, dominant animals had significantly more surviving cluster 9 BrdU+ cells than did subordinate animals but the same as isolate animals (Kruskal-Wallis test for overall difference: $p = 0.0294$; Mann-Whitney test : $p_{D \text{ vs } S} = 0.0022$; $p_{I \text{ vs } D} = 0.1797$, $p_{I \text{ vs } S} = 0.6991$). Over 14 days of survival time, the number of BrdU+ cells approximately doubled twice relative to age-control animals. **C-D.** The number of cluster 10 BrdU+ cells that survived did not change after 7 days (**C**) or 14 days (**D**) of social interaction (Kruskal-Wallis test for overall difference: $p_{7d} = 0.9780$, $p_{14d} = 0.9268$). The number of BrdU+ cells of the 7-day survival groups was similar to that of the age-control animals (**C**), while the number of BrdU+ cells of the 14-day survival groups approximately doubled (D). (I, isolate animals; D, dominant animals; S, subordinate animals; Age Cont, animals in age-control groups; the numbers in parentheses indicate the number of animals examined).


Cell Survival Test (median ± range)
Cluster 9
A. After 7 days
B. After 14 days
# of BrdU+ nuclei
1600
1200
800
400
0
**
Isol
(6)
Dom
(5)
Sub
(6)
Age Cont
(5)
Isol
(6)
Dom
(6)
Sub
(6)
Age Cont
(6)
Cluster 10
C. After 7 days
D. After 14 days
# of BrdU+ nuclei
1600
1200
800
400
0
Isol
(6)
Dom
(5)
Sub
(6)
Age Cont
(5)
Isol
(6)
Dom
(6)
Sub
(6)
Age Cont
(6)

III.2.7. Mitotic Activity in Cell Clusters 9 and 10

Less than 5% of BrdU+ cells were H3P+ cells (i.e., mitotic cells) in clusters 9 and 10 (see Table 4). In all test groups, the number of H3P+ cells in clusters 9 and 10 was not different in isolate, dominant, and subordinate animals after 1 day, 7 days, and 14 days of social interaction (Fig. 20, Table 4; overall difference for 1 day, $p_{cl\ 9} = 0.5015$, $p_{cl\ 10} = 0.6623$; for 7 days, $p_{cl\ 9} = 0.9633$, $p_{cl\ 10} = 0.5316$; for 14 days, $p_{cl\ 9} = 0.1744$, $p_{cl\ 10} = 0.2695$).

Fig. 20 Mitotic activity in cell clusters 9 and 10. Number of H3P+ cells in cluster 9 (**A-C**) and in cluster 10 (**D-F**) after 1 day, 7 days and 14 days of social interaction was not different in isolate, dominant, and subordinate animals. Values are the sum of labeled cells from the two bilateral cell cluster 9s of a brain, and expressed as median ± interquartile range. (I, isolate animals; D, dominant animals; S, subordinate animals; Age Cont, animals in age-control groups; the numbers in parentheses indicate the number of animals examined).


Mitotic Activity (median ± range)
Cluster 9
A. After 1 day
B. After 7 days
C. After 14 days
# of H3P+ nuclei
30
20
10
0
Isol (7)
Dom (5)
Sub (5)
Isol (11)
Dom (11)
Sub (11)
Age Cont (5)
Isol (12)
Dom (10)
Sub (7)
Age Cont (6)
Cluster 10
D. After 1 day
E. After 7 days
F. After 14 days

III.2.8. Subsequent Cell Divisions

To understand the dynamics of cell division in cell clusters 9 and 10 of the crayfish brain, age-control animals were prepared for each of the two cell survival test groups of isolated animals. The age-control groups allowed a comparison between the number of proliferating (BrdU+) cells that was present at a given time point - the incubation of the animal in BrdU – with the number of cells that had survived 7 and 14 days later. Since the number of BrdU+ cells after 7 and 14 days must be the net result of cell divisions going on in the originally proliferating cells counteracted by possible programmed cell death (Harzsch et al., 1999; Schmidt, 2001; Sandeman and Sandeman, 2003), these comparisons are necessary to develop models for the dynamics of cell divisions in olfactory neurogenesis in the brain of juvenile crayfish. The number of BrdU+ cells that survived in cell cluster 9 of isolate animals approximately doubled once after 7 days and twice after 14 days compared to the number in the age-control animals (Table 3; median for age $control_{7d}$ = 215, $isolate_{7d}$ = 398; age $control_{14d}$ = 222, $isolate_{14d}$ = 783). The number of BrdU+ cells that survived in cell cluster 10 increased, but by less than two-fold, after 14 days compared to the number of BrdU+ cells in the age-control animals (Table 3; median for age $control_{14d}$ = 512, $isolate_{14d}$ = 808).

Table 3. Number of BrdU+ cells (minimum, **median**, maximum) in cell clusters 9 and 10, respectively. Numbers in [brackets] represent the number of BrdU+ cells from brains of animals having partial damage to one antennular lateral flagellum. (cl 9, cluster 9; cl 10, cluster 10; n, number of brains examined)

Cell Proliferation Test					
		Isolate	Dominant	Subordinate	Age Cont
1 days	cl 9	82, **137**, 242	93, **166**, 223	114, **173**, 212	NA
	cl 10	261, **371**, 548	387, **411**, 551	394, **448**, 481	
	n	7	5	5	
7 days	cl 9	252, **255**, 318	146, **186**, 260	144, **186**, 196 [140]	NA
	cl 10	432, **550**, 566	307, **394**, 460	366, **427**, 474 [381]	
	n	5	6	5 [1]	
14 days	cl 9	152, **178**, 291	155, **184**, 220 [155]	158 [129, 150, 163]	NA
	cl 10	320, **405**, 439	291, **414**, 452 [372]	398 [314, 342, 392]	
	n	6	4 [1]	1 [3]	
Cell Survival Test					
		Isolate	Dominant	Subordinate	Age Cont
7 days	cl 9	272, **398**, 409	375, **430**, 461 [278]	354, **384**, 531	204, **215**, 248
	cl 10	270, **447**, 624	276, **369**, 550 [233]	307, **375**, 603	387, **443**, 472
	n	6	5 [1]	6	5
14 days	cl 9	578, **783**, 1217	902, **1039**, 1143	441, **727**, 822	182, **222**, 227
	cl 10	457, **808**, 1479	603, **893**, 1200	520, **798**, 1213	380, **512**, 561
	n	6	6	6	6

Table 4. Number of H3P+ cells (minimum, **median**, maximum) in cell clusters 9 and 10, respectively. Numbers in [brackets] represent the number of H3P+ cells from brains of animals having partial damage to one antennular lateral flagellum. (cl 9, cluster 9; cl 10, cluster 10; n, number of brains examined)

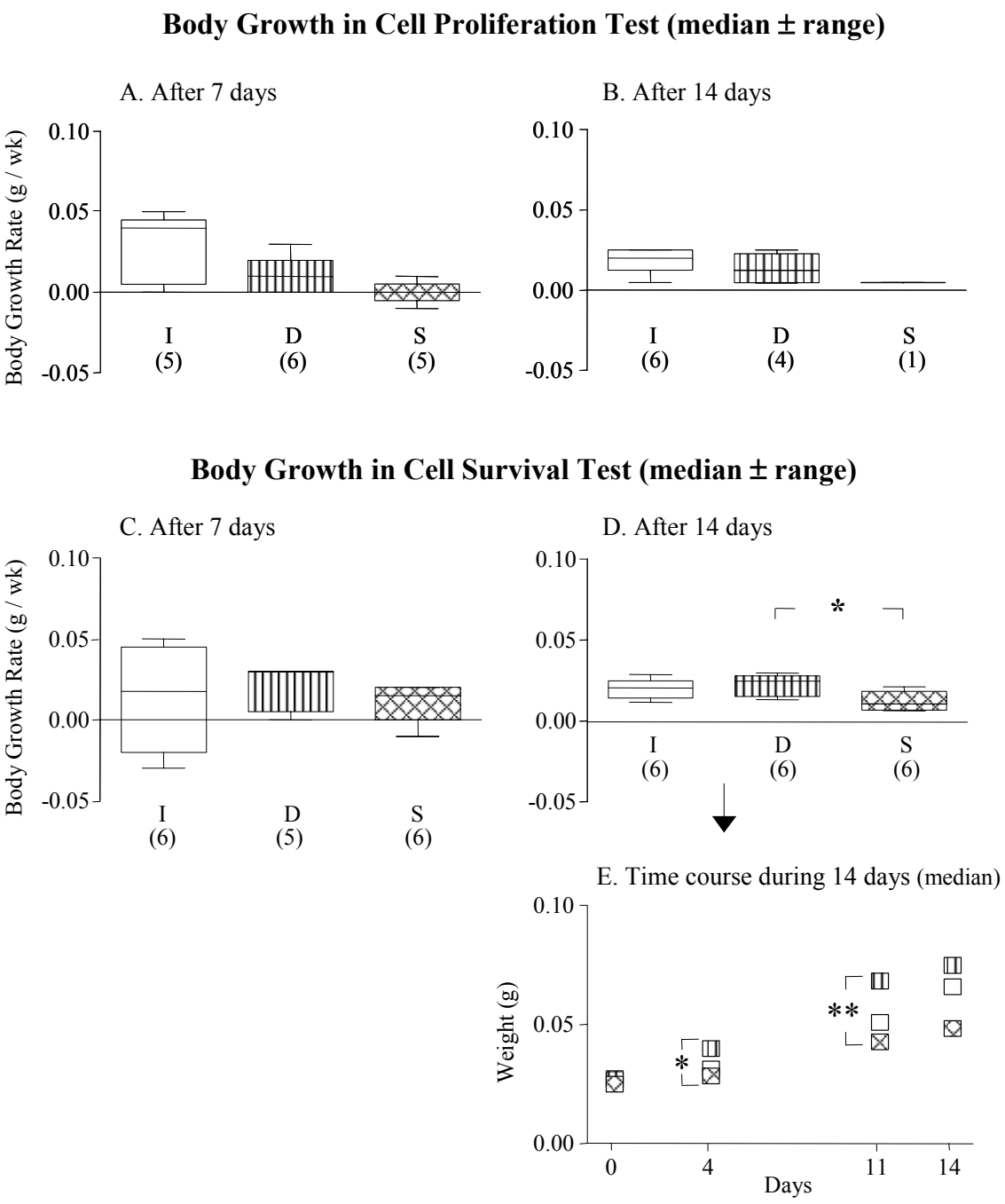
Cell Proliferation Test					
		Isolate	Dominant	Subordinate	Age Cont
1 days	cl 9 cl 10 n	0, **2**, 5 0, **8**, 13 7	1, **3**, 7 3, **9**, 13 5	0, **2**, 12 0, **4**, 16 5	NA
7 days	cl 9 cl 10 n	2, **6**, 14 11, **17**, 28 5	2, **6**, 9 6, **9**, 12 6	4, **5**, 8 [0] 9, **11**, 12 [6] 5 [1]	NA
14 days	cl 9 cl 10 n	0, **4**, 8 5, **11**, 14 6	2, **3**, 5 [2] 61, **10**, 14 [13] 4 [1]	3 [0, 2, 6] 9 [5, 6, 6] 1 [3]	NA
Cell Survival Test					
		Isolate	Dominant	Subordinate	Age Cont
7 days	cl 9 cl 10 n	0, **3**, 8 0, **2**, 4 6	1, **2**, 5 [0] 0, **2**, 4 [2] 5 [1]	0, **4**, 6 0, **3**, 8 6	3, **4**, 10 5, **9**, 13 5
14 days	cl 9 cl 10 n	0, **6**, 13 8, **11**, 18 6	7, **11**, 16 11, **14**, 18 6	1, **7**, 10 7, **13**, 19 6	1, **2**, 10 2, **7**, 17 6

III.2.9. Social Status and Body Growth Rate

When other factors such as sex, body size, and wet body weight were examined, it turned out that the social status of an animal influenced its body growth rate just as it influenced the numbers of surviving cells. The measured body growth rate of an animal was based on wet weight difference from beginning to end of the experimental period. In the 7-day and the 14-day cell proliferation test groups and in the 7-day cell survival test group, no significant differences in the body growth rate of the animals were found (overall difference $p_{7d\ proliferation} = 0.0523$, $p_{14d\ proliferation} = 0.3639$, $p_{7d\ survival} = 0.7254$; Fig. 21A-21C). However, in the 14-day cell survival test group, significant differences were found in the body growth rate of the dominant and subordinate animals (overall difference $p_{14d\ survival} = 0.0304$, $p_{D\ vs\ S} = 0.0152$, $p_{I\ vs\ D} = 0.3939$, $p_{I\ vs\ S} = 0.0649$; Fig. 21D). These differences in body growth rates for animals of different social status occurred despite providing all animals with the same amount of protein-rich food. In the 14 day cell survival test group, where dominant and subordinate animals differed in body growth rate, the body growth rates correlated positively with the number of BrdU+ cells in cell cluster 9 (Pearson correlation $_{cl\ 9} = 0.796$, $p_{cl\ 9} = 0.002$). These results suggest that the effect of social status on neurogenesis might be secondary to the effect on body growth. Alternatively, social status may affect neurogenesis directly and in addition to any effect of body growth. To determine whether cell proliferation and cell survival were influenced by the social status of an animal independent of the effect of body growth, data were subjected to analysis of covariance (ANCOVA; univariate, general linear model) to measure the portion of the variance in cell proliferation and cell survival that

was uniquely associated with social status. Body growth rate of an animal significantly influenced cell survival in cluster 9 after 14 days of social interaction ($p_{cl\ 9} = 0.001$, estimates of effect size being 0.744). However, after removing the effect of body growth, the effects of social status on cell survival in cluster 9 after 14 days of social interaction ($p_{cl\ 9} = 0.036$, estimates of effect size being 0.404) were still significant. Thus, social experience significantly influenced neurogenesis in crayfish, independent of body growth.

Fig. 21 Body growth rate is influenced by the social status. Values are median ± interquartile range of the difference in wet body weight from beginning to end of the experimental period. **A-B.** Body growth rate in the Cell Proliferation Test groups. **A-B.** Body growth rate of animals was not influenced by 7 days (A) or14 days (B) of social interaction (Kruskal-Wallis test for overall difference: $p_{7d} = 0.0523$, $p_{14d} = 0.3639$). **C-D.** Body growth rate in the Cell Survival Test groups. **C.** Seven days of social interaction did not alter the body growth rate of animals (Kruskal-Wallis test for overall difference: $p = 0.7254$). **D.** After 14 days of social interaction, body growth rate was significantly higher for dominant than subordinate animals (Kruskal-Wallis test for overall difference: $p = 0.03$; Mann-Whitney test: $p_{I \text{ vs } D} = 0.3939$, $p_{I \text{ vs } S} = 0.0649$; $p_{D \text{ vs } S} = 0.0152$). **E.** The body growth rate of the animals in D over 14 days of pairing shows that dominant animals generally grew faster than their subordinate partners. The body weight of dominant animals reached a significant difference at 4^{th} day and at 11^{th} day compared to their subordinate partners (Mann-Whitney test: $p_{4th} = 0.0152$; $p_{11th} = 0.0087$). (I, isolate animals; D, dominant animals; S, subordinate animals; the numbers in parentheses indicate the number of animals examined).


Body Growth in Cell Proliferation Test (median ± range)
A. After 7 days
B. After 14 days
Body Growth Rate (g / wk)
0.10
0.05
0.00
-0.05
I
(5)
D
(6)
S
(5)
I
(6)
D
(4)
S
(1)
Body Growth in Cell Survival Test (median ± range)
C. After 7 days
D. After 14 days
I
(6)
D
(5)
S
(6)
I
(6)
D
(6)
S
(6)
*
E. Time course during 14 days (median)
Weight (g)
0.10
0.05
0.00
*
**
0
4
11
14
Days

IV. DISCUSSION

IV.1. A Comma-Shaped Neurogenic Area in Clusters 9 and 10

This dissertation re-defines the morphological features of a life-long proliferation zone in crayfish brain by describing the newborn cells in the tail part of the comma in clusters 9 and 10, the strand structure surrounding them, and the patch of cells near the posterior accessory lobe. That a line of BrdU+ cells emerging from the lateral cluster was previously shown in the brain of adult shore crab and characterized as "string-like" (Schmidt, 1997). However, BrdU+ cells in the tail part of the comma have not been reported for crayfish and lobsters, despite their large cell size compared to the BrdU+ cells in the head part. This is probably because the tail part of the comma emerged ventrally posterior to the cluster core and because it became less obvious, by being sparser, in the adult stage. Individual cells in the patch were lenticular or elongated in shape, suggesting that the patch of cells might be glial in origin (Fig. 8-12). Interestingly, BrdU+ cells in the tail part of clusters 9 and 10, surrounded by the strand structure, joined at the patch of cells (Fig. 8-12), and a similar strand-like structure that surrounds the BrdU+ cells and a patch of glial-like cells are also present in the brain of adult spiny lobster (Schmidt, 2002), suggesting that the strand structure and the patch of cells may be a general feature of neurogenesis in juvenile and adult crustaceans. However, not much is known about the strand structure and the patch of cells. What are the strand structure and the patch? What are their biological functions? The structural aspects of the strand structure surrounding newborn olfactory interneuronal precursors in the crayfish brain are

somewhat similar to the rostral migratory stream guiding newborn olfactory interneuronal precursors in the olfactory bulb of mammalian brains (Ming and Song, 2005). This possibility waits for further examination. It is very strange that the strand structure is positive to tubulin. Tubulin is a component of cytoskeletons, proteins that is known to be 'within' the cell. Furthermore, anti-tubulin did not label tubulin within other cells, around the nuclei, where tubulin is known to be present. No literature has reported the presence of extracellular tubulin in eukaryotic organisms. The strand structure may be a novel structure, or may be axonal projections of other neurons forming the tail of the comma. Furthermore, the strand structure is also positive to anti-H3P, even though the histone-3 protein is known to be present within the nucleus. All these observations suggest that the strand structure may be some sticky structure onto which big proteins such as antibodies unexpectedly bind to. More examination is obviously required to identify the strand structure in the future.

The cells that incorporated BrdU appeared consistently in cell clusters 9 and 10 where the cell bodies of the olfactory local and projection interneurons reside. Therefore, I believe that the proliferation and the survival of the BrdU+ cells in cell clusters 9 and 10 represent neurogenesis. Several markers that are specific to olfactory receptor neurons of lobsters are reported recently, however, whether markers that are specific to brain neuronal precursors not unknown yet (Hollins et al., 2003). However, one cannot completely rule out the possibility that at least some population of BrdU+ cells in these two regions of the brain could be glial cells. Thus, BrdU+ cells were counted following the criteria to eliminate likely glial cells based on cell shape and location (Linser et al.,

1997; Harzsch et al., 1999). Therefore, in the rest of the discussion, the BrdU+ cells will be assumed to represent presumptive neuronal precursors.

The newborn cells that survived in the comma-shaped proliferation zone were faintly labeled after two weeks of survival time due to dilution of the incorporated BrdU after one or more rounds of cell division. Due to subsequent cell division that occurred during the two weeks of survival time, BrdU+ cells in the head part are thought to be pushed outward anteriorly and dorsally toward mature neurons and away from the comma-shaped proliferation zone, a process described in previous studies with lobsters (Harzsch et al., 1999; Schmidt, 2001). These data show that the dynamics of cell divisions differs between clusters 9 and 10. For cluster 10, the simplest model to explain the almost two-fold higher number of BrdU+ cells after a 14 day survival period is that all BrdU+ cells undergo one round of cell divisions within this time period (with some possible losses due to programmed cell death). An approximate doubling of the number of BrdU+ cells after survival times of one to several weeks has also been observed in cluster 10 of embryonic lobsters *Homarus americanus* (Benton and Beltz, 2002), adult shore crabs *Carcinus maenas* (Schmidt, 1997), and adult spiny lobsters *Panulirus argus* (Schmidt, 2001), suggesting a common principle for cell division in this cluster. For cluster 9, the simplest model is that all BrdU+ cells undergo two rounds of cell divisions, one that is complete after a survival time of one week and a second one that is complete after a survival time of two weeks. Dilution of BrdU and mitotic activity among the newborn cells in the tail part of the comma also indicates an occurrence of cell division. However, the actual number of newborn neuronal precursors in the tail part of the comma stayed relatively consistent over two weeks of survival time. It could not be determined

from the experiments executed in this study whether some population of these cells stayed in this region until they became differentiated while another population of them went through apoptotic death or migrated, for example, from the cluster to the tail part or vice versa.

Neuroblasts are present in the brain of several crustaceans during embryonic and larval development; however, the presence of self-proliferative neuroblasts in the brains of juvenile and adult crustaceans is not yet documented (Scholtz, 1992; Gerberding and Scholtz, 2001; Benton and Beltz, 2002). It has been suggested that in the adult lobster brains, a different type of proliferative cell may replace the self-regeneration ability of the neuroblasts seen in the embryo by going through multiple rounds of cell division to produce many olfactory interneuronal precursors (Schmidt, 2001; Benton and Beltz, 2002). In the crayfish *Cherax destructor*, large neuronal stem cells in the embryonic brain are located at the periphery of cell clusters and their presence can be detected up to the fourth molt into adult form (AD IV, adult stage four following terminology in Sandeman and Sandeman, 2003), a developmental stage that had passed during the pre-experimental isolation period in this study. The newborn neuronal precursors in the tail part of the comma observed in this study appear in the same region of cell cluster 10 as described by Benton and Beltz (2002) in lobster embryos. Furthermore, occasional detection of newborn neuronal precursors of large diameter in the tail part of the comma including the patch of cells, being surrounded with the strand, might imply the possible existence of neuroblast-like cells. Interestingly, similar structural features of neuronal proliferation are present in the brain of adult spiny lobsters (Schmidt, 2002). In spiny lobsters, cells of lenticular shape are clustered at one end of the proliferation zone, known

as glial balls (Schmidt, 2002). The glial balls are suggested as presumptive neuroblasts in the brains of adult crustaceans owing to their several distinctive characteristics (Schmidt, 2002). The glial balls are large in diameter compare to other mature neurons, and located at one end of the proliferation zones being surrounded by the strand, and they display continuous mitotic activity (Schmidt, 2002). Studies examining the dynamics of proliferation and survival of newborn neuronal precursors within the proliferation zone including the patch of cells may reveal the dynamics of adult neurogenesis in crayfish brain.

IV.2. Structural Features of Neurogenesis in Comma-Shape Neurogenic Areas in the Crayfish Brain Compared to Olfactory Bulb Neurogenesis in the Mammalian Brain

In the mammalian olfactory bulb, newborn neuronal precursors are born in the subventricular zone, migrate along the rostral migratory stream, and differentiate into distinct types of olfactory interneurons once they reach the main olfactory bulb (Winner et al, 2002; Ming and Song, 2005). A similar sequence of neurogenic events might also occur with newborn neuronal precursors within the comma-shaped proliferation zone in the crayfish brain, although the origin of neuronal precursors and the possibility of migration need to be investigated.

There are distinct structural differences in the mammals and decapoda crustaceans. In the mammalian olfactory bulb, newborn neuronal precursors differentiate into olfactory local interneurons such as granule cells and periglomerular cells, continuously replenishing the population of olfactory local interneurons but not that of

olfactory projection interneurons (Winner et al., 2002). On the other hand, in the brains of crayfish and other crustaceans, both olfactory local and projection interneurons are continuously replenished (Schmidt, 2001; Beltz and Sandeman, 2003; Sandeman and Sandeman, 2003), although the levels of proliferation of olfactory local interneurons greatly reduce in adulthood (Schmidt and Harzsch, 1999; Sandeman and Sandeman, 2000). Furthermore, owing to the structural separation of the two interneuronal clusters by the olfactory lobe and the accessory lobe, newborn neuronal precursors are fated to be either olfactory local or projection interneurons in crayfish brain.

It is interesting to speculate that there could be a structural similarity between olfactory bulb neurogenesis in mammals and the comma-shaped proliferation zone in crayfish. In general, neurogenesis generates neurons that provide new elements for neural circuits and new substrates for synaptic changes during learning and memory. For example, neurogenesis in rat hippocampus is linked to trace memory formation (Shors et al., 2001), and neurogenesis in olfactory brain regions affects responses to and learning of odors in mammals (Rochefort et al., 2002; Lledo and Saghatelyan, 2005) and insects (Scotto-Lomassese et al., 2003). Olfaction, mediated by the olfactory lobes where primary sensory neurons and the two types of olfactory interneurons communicate, plays a prominent role in crayfish and other crustacean animals, including discrimination of social odors (Derby et al., 2001; Horner et al., 2004; Horner and Derby, 2005; Johnson and Atema, 2005). The precise roles of newborn olfactory interneurons are uncertain; however, the changing nature of these olfactory cues and the constant necessity of learning new cues provide a likely reason for their continuous production and turnover.

IV. 3. Social Status-Dependent Changes in Cell Proliferation and Survival

It is perhaps not surprising, then, that neurogenesis is affected by social status and social experience. Social status in crayfish is marked by differences in the behavior of dominant and subordinate animals, including in fighting (Bovbjerg, 1953; Krasne et al., 1997; Herberholz et al., 2001), signaling (Breithaupt and Eger, 2002), sheltering (Herberholz et al., 2003), and reflex avoidance (Song et al., 2000; Issa et al., 2004). Socially dominant animals are more active in exploring, confronting predators, and burrowing than are subordinates (Shively and Kaplan, 1984; Hilakivi-Clarke and Lister, 1992; Herberholz et al., 2003). Dominants use olfactory signals in their urine to communicate with opponents during fights, whereas subordinates refrain from urinating (Zulandt Schneider et al., 1999, 2001; Breithaupt and Eger, 2002). Moreover, the proximity of another animal means that the olfactory cues from that animal will be present and vary in time depending on its activity, distance, and orientation from the receiver. Here physically isolated animals were exposed only to olfactory stimuli from themselves, food, and the mixed and diluted odors from other isolated animals.

These considerations suggest that social olfactory signals experienced by paired animals would induce greater neurogenesis than in unpaired animals that did not experience them. Moreover, the detrimental effects of long-term subordination stress suggest that dominant animals would have greater neurogenesis than subordinates. It was, then, surprising that more proliferation in isolates than in paired animals after 7 days but not after 14 days of pairing, and that dominants and subordinates had similar level of proliferation of new neurons 1, 7, or 14 days after experiencing dominance hierarchies. It

is hard to explain why this transient increase in neurogenesis took place in isolate animals of 7 days cell proliferation test group, but not in isolate animals of 7 day cell survival test group that was treated the same way. There may be some developmentally critical events, related to the age of the animals. Stress, including social stress, reduces neurogenesis in other species (Gould et al., 1997, 1998; Pham et al., 2003), and physical isolation stress in a restricted space reduces neurogenesis in juvenile crayfish (Sandeman and Sandeman, 2000). It may be that the difference between the results of this dissertation and those of Sandeman and Sandeman (2000) is due to differences in experimental design. In their study, juvenile crayfish (a different species – *Cherax destructor*) were individually reared in a small space floating near the water surface, thus restricting exploration and / or locomotor activity. Juvenile crayfish in this study were reared in a similarly small horizontal space; however, they were provided with a relatively large vertical space, having their own shelter and gravel as substrate at the bottom, and above having water plants to climb up and / or to eat. In the present instance, all animals were placed into this restricted space since hatching, so the level of neuronal proliferation in the animals examined in this study is likely to be standardized if decreased by close confinement. It is possible that dominant-subordinate differences in proliferation may arise if close confinement is removed by pairing animals in larger, richer environments.

After 14 days of social pairing, the effects of social experience on cell survival emerged. Dominant-subordinate differences appeared in the survival of proliferating neurons in cluster 9, but not in cluster 10 (Fig. 19). Of the local interneurons that were born in cluster 9 of paired animals, significantly fewer survived in subordinates than in

dominants after 14 days of pairing. This result leads to a speculation that in the longer term, socially dominant crayfish may be better able to process chemosensory signals than subordinates. While the features of olfactory processing controlled specifically by these local and projection interneurons are known (Sandeman and Mellon, 2001; Schachtner et al., 2005), the functional implications of changes in the neurogenesis of local interneurons needs to be identified.

These changes may be related to the process of maintaining dominance hierarchies. The experience of prolonged and continuous social subordination may result in suppression of cell survival. Increased survival of newborn neuronal precursors due to acquiring dominant status has also been reported in rat hippocampus (Kozorovitskiy and Gould, 2004). During two weeks of social interaction, only subordinates may be under the suppressive effect of stress on cell survival as they avoid the dominant and defend against attacks when they cannot. Dominant animals, which move freely about regardless of the presence of their subordinate partners, may be resistant to this suppressive effect (Kozorovitskiy and Gould, 2004). In addition, prolonged social subordination is often accompanied with bodily injury including their externally exposed chemosensory organ, the lateral flagella of the antennules. Indeed, subordinate animals in the 14-day cell proliferation test suffered the most damage to their lateral flagella. Complete ablation of one lateral flagellum drastically reduces cell proliferation in adult shore crab (Hansen and Schmidt, 2001). Thus, it is expected that in nature, cell proliferation is likely to be also suppressed in subordinate crayfish following injury as a consequence of a long-term social subordination.

Kozorovitskiy and Gould (2004) measured the levels of circulating corticosterone and found that stress measures did not correlate with the social status of an animal (Kozorovitskiy and Gould, 2004). In crustaceans, hypoxic stress, caused by taking the animal out of the water for a few hours, results in an increase in the level of crustacean hyperglycemic hormone (CHH) in the hemolymph (Webster, 1996; Chang et al., 1998), and thus CHH has been suggested to be a putative stress hormone in crustaceans. Measurement of the CHH level of the animals used in this study was not attempted, however, due to the correlation of CHH level with the molt cycle (Chung et al., 1999). Yet, careful measurement of CHH level, probably using adult crayfish whose molt cycle is less influenced by minor body damage, in correlation with social experience of crayfish, may clarify whether a reduction in cell proliferation and cell survival is possibly due to the stress of experiencing dominance hierarchy formation and social subordination.

Forming and maintaining a dominance hierarchy is a complex phenomenon that occurs naturally in groups of animals, and it involves learning and memory formation of a variety of sensory (olfactory, visual, and tactile) cues and motor outputs. Accumulating studies document that proliferation and survival of newborn neuronal precursors may represent the mechanism of learning and memory (Gould et al., 1999a, 1999b; Shors et al., 2001). Thus, learning of olfactory signals in urine released toward each other while a pair of crayfish fight, which is indispensable for the formation of a dominance hierarchy (Breithaupt and Eger, 2002; Zulandt Schneider et al., 2001), may regulate the survival of olfactory interneuronal precursors in the brains of dominant and subordinate crayfish. Increased survival of olfactory interneuronal precursors may improve memory formation

in dominant crayfish, and thus increase dominants' chances of survival in nature. For the best chances of survival in nature, peripheral neurogenesis is likely to coordinate somehow with the dynamics of central neurogenesis. Thus, it is of great interest to see whether and how proliferation, survival, and differentiation of newborn olfactory sensory neuronal precursors in the lateral flagella of the antennules, the 'nose' of crayfish, keep pace with the pace of proliferation and survival of olfactory interneurons in the brain which I have shown to be controlled by social experience. The rule of the survival of the fittest may apply to crayfish with the best 'nose'.

Part of this study was previously published in abstract form in the annual meetings for the Society for Neuroscience (Song et al., 2004, 2005).

Discussion

Harsh life experience to the degree of threatening one's survival is no doubt stressful. When it is prolonged, the consequences can be detrimental even causing physical and mental illness. Inescapable social stress can change the circuitry of our brains, and thus our behavior. Thus, this dissertation examined the effect of inescapable social stress on animal's behavior and animal's brain neurogenesis with crayfish *Procambarus clarkii*.

The first chapter of this dissertation demonstrated changes in behavior to unexpected touch after the experience of dominant-subordinate relationship. The dominant animals moved about freely, consistently displayed aggressive behaviors, and orienting responses to unexpected touch regardless of the social context at the time of testing. Isolate animals behaved in the same manner as dominant animals. On the other hand, the subordinate animals under inescapable subordination stress, displayed submissive behaviors and avoidance responses to unexpected touch. In social contexts where inescapable subordination stress was lessened or removed, the same subordinate animals displayed aggressive behaviors and orienting responses to unexpected touch. These findings suggest that neural circuits controlling the two different movement patterns, i.e., orienting and avoidance, may be reciprocally activated depending on the social status as well as on the social context. If so, life-long inescapable subordination stress may reduce the plasticity of the neural circuits in subordinate animals, resulting in permanent submissive behaviors including avoidance response. Previous attempts to test this possibility failed because no subordinate crayfish survived after living together with

their dominant partners for 2 weeks due to serious bodily damage. This limitation could be resolved by providing the optimal living space for the pairs: small enough for a stable dominance hierarchy formation, but big enough for subordinates to have minimal body injuries.

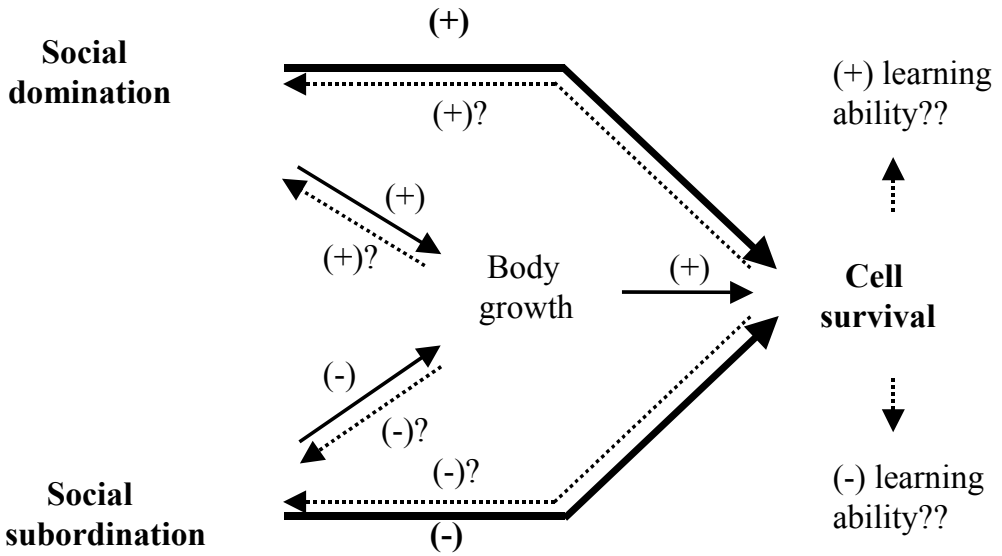
The second chapter of this dissertation examined social status-dependent changes in cell proliferation and cell survival in the brain regions retaining life-long plasticity. For the continuity of dominant-subordinate relationship, juvenile crayfish with soft exoskeletons were used. Life-long neurogenesis occurred in a comma-shaped proliferation zone in the brain regions involved in olfaction. The number, size, and shape of newborn neuronal precursors differed depending on the regions of the comma-shaped proliferation zone and on the survival time. With longer survival time of 14 days, more neuronal precursors were surviving in the head of the comma than in the tail of the comma, and the size and shape of surviving newborn neuronal precursors were similar to those of mature olfactory interneurons. Thus, it may be that the biological function of newborn neuronal precursors may differ depending on the regions of the cell proliferation zone. For example, neuronal precursors in the head of the comma may survive and differentiate to functional olfactory interneurons, i.e., expression of neurotransmitter and incorporation into the olfactory circuits, as it has shown in other crustaceans (Schmidt, 2001; Sullivan and Beltz, 2005), while neuronal precursors in the tail part may transiently survive and then die and / or migrate into other regions of the comma, such as into the head part. It is of great interest whether these functional and structural features of the neurogenic olfactory pathway of invertebrates, such as crayfish may extend to the neurogenic olfactory pathway of vertebrates such as birds and mammals. The newborn

neuronal precursors in the strand structure and the patch of cells in the crayfish brain, although their function in neurogenesis is yet unknown, might be candidates for such a parallel.

After a long-term experience of dominant-subordinate relationship (14 days), the survival of newborn olfactory interneuronal precursors was significantly reduced in subordinate animals compared to the dominant partners. The effects of longer social subordination stress are unclear. It is likely that subordination stress including frequent damages to the lateral antennular flagellum would suppress cell proliferation and survival in the brain of crustaceans (Hansen and Schmidt, 2001). Subordinate animals, having significantly fewer surviving newborn neuronal precursors in the brain, may have reduced learning ability that ultimately endangers their survival and reproductive success (Fig. 22). This hypothesis is speculative at this stage because of the lack of experimental data showing how many surviving newborn cells ultimately differentiate into neurons that are incorporated into functional circuitry in the brain. Other studies have shown that not all, but a significant portion of the newborn cells, differentiates into neurons expressing neuron-specific chemicals such as neurotransmitter (Schmidt, 2001; Sullivan and Beltz, 2005). Further studies testing whether subordinate animals possess poor learning ability depending on the degree of subordination stress, if any, would help better understand the neurobiology of social status.

Fig. 22 Suggested model for social status and neurogenesis. Thick straight lines summarize the experimental results shown above, while dashed lines suggest the hypothetical relationships. For crayfish that were individually reared since hatching, experience of social domination for 14 days enhances the cell survival of olfactory local interneuronal precursors, while experience of social subordination for 14 days reduced it. Body growth of animals, affected by social status, is positively correlated with the level of cell survival of olfactory local interneuronal precursors. Although it remains to be tested, status-dependent body growth and cell survival are likely to influence social status formation and maintenance, and furthermore, the learning ability of crayfish.

A. Suggested Model



Interestingly, the continuity and duration of dominance fights appear to influence the level of neurogenesis. Other studies that examined social status-dependent neurogenesis support the idea that the degree of subordination stress correlates with the continuity and duration of repeated dominance fights. Brief daily interactions (5-30 min / day) significantly enhance proliferation and survival of olfactory projection interneuronal precursors in dominant and subordinate animals compared to isolate controls only when size-'matched' juvenile American lobsters, *Homarus americanus*, were paired (Mazzarella, 2003; Kim, 2004). Similarly, brief daily interactions (60 min / day) enhance cell proliferation in the hippocampus in dominants and subordinates compared to isolate controls between pairs of adult mice (Fiore et al., 2005). On the other hand, longer daily interactions (6.5 hr / day) reduce cell proliferation in the hippocampus of subordinates compared to dominants between pairs of adult birds (Pravosudov and Omanska, 2005). When juvenile crayfish live together in a pair, the process of forming and maintaining dominance hierarchies reduce survival of newborn neuronal precursors in subordinate members (this study). A similar reduction in cell survival also occurs in the hippocampus of subordinates in groups of adult rats (Kozorovitskiy and Gould, 2004). When three juvenile crayfish live together, cell proliferation but not cell survival is decreased in two subordinate animals (Pelz, 2001). In Pelz's study (2001), by rearing three animals together and defining the alpha animal as the dominant and the other two beta and gamma animals as the subordinates, the degree of subordination stress might have been weaker to produce the reduction in cell survival observed in this study from pair-wise pairing. These results argue for a degree of subordination stress in the duration of daily

intermittent pairing necessary to reflect the process of social subordination that reduces cell proliferation and/or survival of newborn neuronal precursors.

The life span of crayfish *Procambarus clarkii* is reported to be approximately 2-3 years and body size at maturity ranges approximately 6-12 cm. (Holdich, 1992). Upon hatching, crayfish hatchlings molt twice, approximately once a week, into juvenile stage. The juvenile crayfish examined in this dissertation were 5-9 weeks old, ranging 1.1-2.2 cm in body size. At this developmental stage, greatly more newborn neuronal precursors were proliferating in the comma shape proliferation zone, probably to support the brain development and to structure the brain circuitry. Thus, the effect of social status on the survival of olfactory interneuronal precursors in the brain of juvenile crayfish may result in a permanent change in brain plasticity and circuitry through adulthood, or may be overridden by growth demands. More studies examining neurogenesis in the crayfish brain throughout all developmental stage, and studies examining the effect of early life experience on the level of neurogenesis at adult stage will resolve the issue.

The cellular mechanisms underlying the formation and maintenance of a dominance hierarchy is unknown. This dissertation provides additional evidence that social experience not only changes animal's behavior but also changes animal's brain neurogenesis. This dissertation also reveals, for the first time, additional cell proliferation zone in the crayfish brain, embedded in the strand structure to the patch of cells. What are the cellular mechanisms of a dominance hierarchy formation and maintenance? What are the strand structure and the patch of cells? What are the

biological roles of newborn neuronal precursors in the crayfish brain? All these questions need to be answered in the future.

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