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Measuring *inotocin receptor* gene expression in chronological order in ant queens

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ABSTRACT

In vertebrates and invertebrates, oxytocin/vasopressin-like peptides modulate a variety of behaviors. The recent discovery of the gene and receptor sequences of inotocin, the insect ortholog of oxytocin/vasopressin, opens new opportunities for understanding the role of this peptide family in regulating behaviors in the most populated class of living animals. Ants live in highly organized colonies. Once a year, they produce future queens that soon leave the nest to mate and found new colonies. During the first months of their lives, ant queens display a sequence of behaviors ranging from copulation and social interactions to violent fighting. In order to investigate the potential roles of inotocin in shaping queen behavior, we measured gene expression of the *inotocin receptor* in the heads of *Lasius niger* ant queens at different points in time. The highest levels of expression occurred early in queen life when they experience crowded conditions in their mother nests and soon thereafter set out to mate. Inotocin could thus be involved in regulating social and reproductive behaviors as reported in other animals. While oxytocin and vasopressin are also involved in aggression in mammals, we found no direct link between these behaviors and *inotocin receptor* expression in *L. niger*. Our study provides a first glimpse into the roles the inotocin receptor might play in regulating important processes in ant physiology and behavior. Further studies are needed to understand the molecular function of this complex signaling system in more detail.

1. Introduction

The existence of oxytocin/vasopressin-like peptide signaling has been documented in both vertebrates and invertebrates, and is believed to have evolved from an ancestral peptide before the split between Proto- and Deuterostomia, 640–760 million years ago (Acher and Chauvet, 1995; Douzery et al., 2004). Vertebrates have two peptide homologs, oxytocin and vasopressin, whereas invertebrates usually have only one. In either case, receptors belong to the G protein-coupled receptor family (Beets et al., 2013; Donaldson and Young, 2008; Gimpl and Fahrenholz, 2001). Oxytocin/vasopressin-like peptides regulate a wide range of peripheral physiological processes. In particular, oxytocin plays a major role in stimulating uterus contractions during parturition in mammals (Gimpl and Fahrenholz, 2001), and in annelids and possibly also mollusks, oxytocin/vasopressin-like peptides are involved in egg-laying behaviors (Fujino et al., 1999; Oumi et al., 1996; Van Kesteren et al., 1995; Wagenaar et al., 2010). The peptide family also has a number of important central effects in the invertebrate and vertebrate brain (Beets et al., 2013; Feldman et al., 2016; Gimpl and Fahrenholz, 2001; Gruber, 2014). For instance, both vertebrate oxytocin (Borrow and Cameron, 2012; Goodson, 2013) and invertebrate oxytocin/vasopressin-like peptides (Gruber, 2014) have been linked to reproductive behaviors in several species. In rodents (Dore et al., 2013; Young and Wang, 2004), birds (Goodson et al., 2009; Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012) and even humans (Carter et al., 2009; Hurlemann et al., 2010; Kosfeld et al., 2005; Rilling et al., 2012), the peptide appears to be involved in mediating a range of social interactions such as pair bonding and cooperation. In contrast, vasopressin and oxytocin also seem to modulate certain aggressive behaviors in both rodents and humans (Pagani et al., 2013).

Oxytocin/vasopressin signaling and function have been primarily studied in vertebrates. Surprisingly, the role of these peptides in shaping behavior and physiology remains largely unknown in insects, which represent 58% to 80% of all animal species (Scudder, 2009). The oxytocin/vasopressin-like peptide in insects was first discovered in the locust *Locusta migratoria* (Orthoptera) (Proux et al., 1987). Later, the genes coding for this same oxytocin/vasopressin-like peptide and its G protein-coupled receptor were identified in the genome of the red flour beetle *Tribolium castaneum* (Coleoptera) (Aikins et al., 2008; Stafflinger et al., 2008). Stafflinger et al. (2008) named the peptide inotocin, for insect oxytocin/vasopressin-like peptide, and showed that inotocin strongly activated its receptor in vitro. The gene expression of the

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inotocin preprohormone matched that of the receptor in different body parts and developmental stages in *T. castaneum* (Stafflinger et al., 2008). To date, the gene sequences of inotocin and its receptor have been identified in the genomes of over 100 insect species from at least 20 representative orders (Gruber and Muttenthaler, 2012; Liutkeviciute et al., 2016; Stafflinger et al., 2008). However, inotocin peptides and receptors are absent from the genome sequences of certain orders, namely Diptera and Lepidoptera, suggesting that inotocin signaling is restricted to basal insect lineages (Liutkeviciute et al., 2016; Stafflinger et al., 2008).

Ants are excellent biological models for studying the functional role of inotocin, as they display diverse and remarkable behaviors. They live in highly organized societies characterized by reproductive division of labor, whereby one or a few individuals (the queens and the males) specialize in reproduction, whereas the others (the workers) forego their own reproduction to participate in cooperative tasks such as building the nest, collecting food, rearing the young or defending the colony (Hölldobler and Wilson, 1990). Typically, once a year mature colonies produce new queens and males that soon leave the safety of their mother colony to mate during large nuptial flights. After mating, males die, while newly mated queens land on the ground and start searching for an adequate nesting site to establish their future colony. Once queens have settled in their new nest, they start to lay eggs and will continue doing so for the rest of their decade long lives while never mating again (Boomsma, 2013, 2009, 2007). Young mated ant queens can either found their colony alone (haplometrosis) or in cooperation with one or more unrelated co-founding queens (pleometrosis) (Bernasconi and Strassmann, 1999; Hölldobler and Wilson, 1977). Pleometrotic colony foundations are usually more competitive and survive better compared to haplometrotic foundations. However, pleometrosis is a risky endeavor since in most species queen cooperation breaks down at the time of emergence of the first workers, as queens engage in violent fights where only the winner survives and continues to head the colony alone.

We measured, to our knowledge for the first time, the gene expression levels of the *inotocin receptor* during insect reproductive, cooperative and aggressive behaviors, using *Lasius niger* ant queens as a biological model. In this species, queens can found their colony either by haplometrosis or pleometrosis, allowing us to investigate both scenarios. We compared the expression levels of the *inotocin receptor* at specific points in time, from sexual maturity to one year after mating, i.e. when queens sequentially engage in mating, social interactions, and fighting. Relative mRNA levels were measured by quantitative PCR in virgin queens, newly mated queens, haplo- and pleometrotic founding queens and one year old queens. Expression levels were quantified in the queen head, to exclude any potential background noise that could arise from involvement of inotocin in physiological processes in other parts of the body.

2. Materials and methods

2.1. Queen collection and rearing

Virgin and freshly mated queens were collected in Brussels, Belgium, during June and July 2015 and 2016. We first collected virgin queens by excavating three colonies during the course of one day, approximately two weeks before the nuptial flight (referred to as *young virgin queens* hereafter). At the onset of the nuptial flight, we collected virgin queens preparing to take off from the entrances of three different colonies (referred to as *flight ready virgin queens*) (Fig. 1). Young virgin queens ($n = 6$) and flight ready virgin queens ($n = 6$) were snap-frozen in liquid nitrogen immediately after collection. During the nuptial flight, when queens start landing after mating, we collected a large batch of freshly mated queens on the ground. A first, randomly selected, group of these ($n = 6$) were killed by snap-freezing within two hours (time needed to complete the sampling and bring queens back to the laboratory) after collection (referred to as *freshly mated queens*) (Fig. 1). The rest were installed into artificial nests (glass tubes divided into three parts: water, a cotton plug and a dry area for the actual nest space) either by themselves (haplometrosis) ($n = 30$) or two by two (pleometrosis) ($n = 36$). Pleometrotic queen pairs were assigned at random. After the emergence of the first workers (ca. 30 days after the mating flight) colonies were given ad libitum sugar water and mealworms.

The nesting haplometrotic and pleometrotic founding queens were randomly assigned to separate groups that were snap-frozen either, one, 20, 40 or 80 days after mating (n = 6 queens in each group) (Fig. 1). For haplometrotic queen nests, we added an extra group consisting of queens that were killed one year after mating ($n = 6$). For pleometrotic queen nests, we added two extra groups in which queens were killed either during or one day after their fight for colony rule (each, $n = 6$). Because we were interested in measuring gene expression according to queen behavior, and not by a comparison between winners and losers during fights, we sampled only one of the two foundresses from pleometrotic foundations. Before the fights (i.e., one, 20 and 40 days after mating) and during the fights, the queens used for gene expression measurements were selected at random, as it was not straightforward to predict which queen would win the fight (Aron et al., 2009; Holman et al., 2010; Sommer and Hölldobler, 1995). After the fight (i.e., one day after the fight and 80 days after mating), the queen used was invariably the winner, as the loser always died during or soon after the fights, precluding their use for gene expression quantification.

After snap-freezing, all queens were stored at − 80 °C until further use.

2.2. RNA extraction and reverse transcription

We chose to use whole heads and not only brains as the latter are small and timely to dissect which might jeopardize RNA integrity due to degradation during dissection. Queens were removed from storage at − 80 °C and their heads were immediately homogenized in TRI Reagent® Solution (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a Mixer Mill MM301 (Retsch GmbH, Haan, Germany) using 2.8 mm Precellys® zirconium oxide beads (Bertin Corp, Rockville, Maryland, USA). Total RNA was then extracted following the TRI Reagent® Solution RNA isolation protocol (TRI Reagent® Solution, RNA/DNA/Protein Isolation Reagent, RNA Isolation Procedure, 2010, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The amount of extracted RNA was measured with a Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and RNA quality and integrity were verified using Nanodrop™ absorption ratios and band inspection after electrophoresis on a 2% agarose gel.

Each individual queen head RNA sample was then treated with DNase I, Amplification Grade (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove traces of genomic DNA (Deoxyribonuclease I, Amplification Grade, Protocols, 2002, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reverse transcription of the mRNA was carried out on 160 ng of DNase I treated total RNA using Oligo$(dT)_{20}$ Primer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and SuperScript™ III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's protocol (SuperScript™ III Reverse Transcriptase, First-Strand cDNA Synthesis, 2004, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The reverse transcription program consisted of 60 min at 50 °C, followed by 15 min at 70 °C. To control for genomic DNA contamination in the following quantitative PCR step, we included a no reverse transcription (no RT) control containing water instead of reverse transcriptase for each sample. cDNA samples and no RT controls were diluted 10 times in Buffer AE (Qiagen, Venlo, Netherlands) and stored at − 20 °C until gene expression analysis.