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Immediate early gene activation throughout the brain is associated with
dynamic changes in social context

Cait M. Williamson\(^a\), Inbal S. Klein\(^a\), Won Lee\(^a\) and James P. Curley\(^{a,b}\)

\(^a\)Department of Psychology, Columbia University, New York, NY, USA; \(^b\)Department of Psychology, UT Austin, Austin, TX, USA

ABSTRACT
Social competence is dependent on successful processing of social context information. The social opportunity paradigm is a methodology in which dynamic shifts in social context are induced through removal of the alpha male in a dominance hierarchy, leading to rapid ascent in the hierarchy of the beta male and of other subordinate males in the social group. In the current study, we use the social opportunity paradigm to determine what brain regions respond to this dynamic change in social context, allowing an individual to recognize the absence of the alpha male and subsequently perform status-appropriate social behaviors. Replicating our previous work, we show that following removal of the alpha male, beta males rapidly ascend the social hierarchy and attain dominant status by increasing aggression towards more subordinate individuals. Analysis of patterns of Fos immunoreactivity throughout the brain indicates that in individuals undergoing social ascent, there is increased activity in regions of the social behavior network, as well as the infralimbic and prelimbic regions of the prefrontal cortex and areas of the hippocampus. Our findings demonstrate that male mice are able to respond to changes in social context and provide insight into the how the brain processes these complex behavioral changes.

Introduction
Organization into dominance hierarchies is a fundamental feature of group social behavior across species, including non-human primates (Muller & Wrangham, 2004; Sapolsky, 1993), cichlid fish (Grovenick, Clement, & Fernald, 2007; Huffman, Hinz, Wojcik, Aubin-Horth, & Hofmann, 2015; Oliveira & Almada, 1996), naked mole rats (Holmes, Goldman, & Forger, 2008), honey bees (Kucharski, Maleszka, Foret, & Maleszka, 2008), mice (Wang et al., 2011; Williamson, Lee, & Curley, 2016), and humans (Zink et al., 2008). Individuals form these dominance structures through a complicated appraisal of their social context in order to ascertain their position relative to that of the other individuals within their social network (Curley, 2016b; Fernald, 2014; Grovenick et al., 2007; Oliveira, 2009). There has been characterization of the complex behavioral features of the formation and maintenance of dominance hierarchies (Chase & Seitz, 2011; Chase, Tovey, Spangler-Martin, & Manfredonia, 2002; Curley, 2016b; Williamson, Lee, et al., 2016; Williamson, Lee, Romeo, & Curley, 2017), as well as identification of the neural correlates associated with social status in stable social hierarchies (So, Franks, Lim, & Curley, 2015; Wang et al., 2011; Williamson, Franks, & Curley, 2016; Zerubavel, Bearman, Weber, & Ochsner, 2015; Zink et al., 2008).

Although hierarchies are commonly stable, there often occurs times when individuals change in social rank. One particularly salient example of this is when a power vacuum emerges at the top of a hierarchy following the removal or deposition of the alpha individual. When such social opportunities occur, subdominant animals typically rapidly ascend to the alpha position. Such behavior has been observed experimentally in hierarchies of both cichlid fish (Maruska & Fernald, 2010) and CD1 outbred mice (Williamson, Romeo, & Curley, 2017) associated with changes along the hypothalamic-pituitary-gonadal (HPG) axis. Ascent to dominant status in cichlid fish is also associated with increased immediate early gene expression in several regions specific to fish social behavior (Burmeister, Jarvis, & Fernald, 2005; Maruska, Zhang, Neboori, & Fernald, 2013). However, there has been no comprehensive, whole brain analysis of the neural response to changes in social context in mammals. This ability to process this dynamic social context information and behave in a socially competent manner when the structure of a social hierarchy shifts is critical for successful social living.
The “Social Behavior Network” (SBN) is a bidirectional circuit of brain regions associated with multiple forms of social behavior (i.e., aggression, sexual behavior, communication, social recognition, affiliation and bonding, parental behavior, and social stress responses) across species (Goodson, 2005; Newman, 1999). This network was first described to include the medial amygdala (meA), the bed nucleus of the stria terminals (BNST), the lateral septum (LS), the medial preoptic area (mPOA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), and the periaqueductal grey (PAG) (Newman, 1999). These brain regions are thought to be the core of the social brain, with much supporting evidence for their role in regulating relatively simple social behavior (see Goodson, 2005 for a comprehensive review). However, for complex social behaviors, such as the formation, maintenance, and dynamic adjustment of social hierarchies, which are reliant on an individual’s ability to perceive changes in their social environment, it is important to understand how activity within the SBN is modulated and complemented by brain regions associated with executive functioning (i.e., prefrontal cortex Wang et al., 2011; Zink et al., 2008) and memory (i.e., hippocampus Noonan et al., 2014; Williamson, Franks, et al., 2016).

In previous studies, we have demonstrated differential gene expression throughout the brains of outbred CD1 mice of different social rank living in linear hierarchies, specifically in the medial amygdala, central amygdala, medial preoptic area (So et al., 2015) and in the whole hippocampus (Williamson, Franks, et al., 2016). We have shown that within minutes of the removal of the dominant male from a social group, the subdominant male exhibits increased aggression as well as rapid changes in GnRH gene expression in the medial preoptic region of the hypothalamus (Williamson, Romeo, et al., 2017). In the current study, we aimed to generate a map of immediate early gene activity throughout the SBN and areas related to the monitoring of social context and social memory to assess how the brain of subdominant animals responds to a changing social context when a social opportunity to ascertain alpha status arises. Specifically, we assessed the pattern of Fos immunoreactivity in subdominant mice in response to the removal of the alpha male (a dynamic social change) and compared this neural response to that of subdominant mice living in a stable social system.

**Methods**

**Subjects and housing**

A total of 48 male outbred CD1 mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 2 weeks in standard sized cages prior to the behavioral experiment. Throughout the study, mice were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30–50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red light (dark cycle) on at 1200 hours. All mice were individually and uniquely marked by dying their fur with a blue, nontoxic, non-hazardous animal marker (Stoelting Co.). These marks remain for up to 12 weeks and only require one application, enabling each animal to be visually identified throughout the study. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No: AC-AAAP5405).

**Behavioral manipulation**

To determine Fos activation associated with social ascent, we performed a social opportunity manipulation comparing subdominant individuals in socially stable groups to those ascending in a hierarchy from subdominant to dominant status. This procedure is similar to that previously described (Williamson, Romeo, et al., 2017). At 9 weeks of age, 4 groups of 12 mice were placed into custom built vivaria (length 150 cm, height 80 cm, width 80 cm; Mid-Atlantic; Supplemental Figure 1). The vivarium was constructed as previously described (Williamson, Lee, et al., 2016). Each vivarium consists of an upper level consisting of multiple shelves covered in pine bedding and a lower level consisting of a series of nest-boxes filled with pine bedding connected by tubes. Mice can access all levels of the vivarium via a system of ramps and tunnels. Standard chow and water were provided ad libitum at the top of the vivarium, encouraging movement and exploration of all the shelves. Social groups were introduced into the vivarium directly before onset of the dark cycle on Day 1. There were 4 social groups, each with 12 mice per group. Each group was paired with one other group for experimental control and counter-balancing purposes. For example, when group 1 was experimentally manipulated, group 2, its paired group, served as the control condition. Live behavioral observations were conducted each day during the dark cycle. These observations consisted of trained observers recording all instances of fighting, chasing, mounting, subordinate posture, and induced-flee behaviors. Each trained observer was responsible for observing one cohort at a time, so observers were entirely focused on one group during their observation. The identity of the dominant and subordinate individuals in each interaction were recorded using all occurrence sampling. Data was collected directly into electronic tablets and uploaded live to a google spreadsheet. Supplemental Table 1 contains...
an ethogram of the behaviors recorded. Live observations were conducted for 2 hours during the first four hours of the dark cycle each day on Days 1–4 of group housing. At the end of Day 4, it was verified that a dominance hierarchy had emerged in each group, and the identity of the alpha and beta male in each group was determined. The presence and linearity of the hierarchies was confirmed through calculating Landau’s modified h’ values, and the identity of the alpha and beta male was confirmed using Glicko scores and examination of the sociomatrix of wins and losses. These analyses are described below in the statistical analysis section. On Day 5, at the onset of the dark cycle, the alpha male from one of the paired cohorts was removed from the vivarium and placed in a standard cage with food and water. In the other paired cohort, the alpha male was sham-removed, which entailed an experimenter opening the Perspex windows to the vivarium, placing their hand in the vivarium, and reaching towards the alpha mouse but not removing it from the vivarium. This condition, which does not involve removing any mouse from the social group, controls for behavioral changes that may be occurring in response to a non-social disturbance to the environment. Live behavioral observations occurred for the period directly following the removal or sham-removal. Ascending males were confirmed as the individual who won most aggressive contests post-alpha removal without consistently losing to other individuals. Ninety minutes after this ascending individual had won three fights, the ascending male was removed from the alpha removal group and the non-ascending subdominant male was removed from the sham-removal group.

Following removal from the social group, mice were anesthetized with ketamine/xylazine and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were stored at 4°C in 4% paraformaldehyde for the first six hours following perfusion and then switched to a 30% sucrose solution. Following the perfusions, the alpha male who had been removed in the social ascent condition was returned to his social group. Alpha males always retained their alpha status on return to the social group. This procedure was repeated at four day intervals for a total of six “removals”. Manipulations were counter-balanced between paired cohorts (i.e., one vivarium had alpha removal for removals 1, 3, and 5, and sham removals for removals 2, 4, and 6, and the opposite was true for the paired vivarium). Each removal/sham-removal decreased the size of the social group by 1, resulting in N = 12 (removal one), N = 11 (removal two), N = 10 (removal three), N = 9 (removal 4), N = 8 (removal 5), N = 7 (removal 6). This design yielded 12 mice per group from two groups: beta male/alpha removed (ascending males) and beta male/alpha remained (sham-removal subdominants). See Figure 1 for a schematic of the behavioral manipulation.

**Immunohistochemistry**

Brains were stored in 30% sucrose in 0.1 M PB at 4°C until slicing. Perfused whole brains were sliced coronally into 40 μm sections and stored in 0.1 M PB azide until processing according to the avidin–biotin procedure, using the Vectastain ABC Elite peroxidase rabbit IgG kit (Vector Laboratories, Burlingame, CA). Free-floating sections were transferred into wells and washed three times in 0.1 M PB for five minutes each rinse. The sections were then washed once in hydrogen peroxide for five minutes and then washed three times in PBT for five minutes each rinse. The sections were then placed in a solution of 2% Normal Goat Serum (NGS, Vector Laboratories) in 0.1% Triton-X in 0.1 M PB (PBT) for an hour, and then incubated in primary Fos rabbit polyclonal IgG (Santa Cruz, USA, SC-52) at a concentration of 1:5000 overnight at 4°C with 2% NGS block. The next day, the sections were washed 3 times in PBT for 5 minutes each rinse and then incubated in biotinylated anti-rabbit IgG (Vecstastain ABC Kit, Vector Laboratories) at a concentration of 1:200 in PBS for 1 hour. Once the hour was complete, sections were once again washed 3 times in PBT for 5 minutes each rinse. Sections were then incubated for 1 hour in an avidin–biotin–peroxidase complex in 0.1 M PBT (A and B solutions of the Vectastain ABC Kit, Vector Laboratories) at a concentration of 40 ul A: 40 ul B: 10 ml PBT and then washed 3 times in 0.1 M PBS for 5 minutes each rinse. Fos immunoreactivity was visualized by incubating the sections in 0.02% 3,3′-diaminobenzidine (DAB) solution for 2–4 minutes. Sections were then washed once for 1 minute in 0.1 M PBS and then washed 3 times in 0.1 M PBS for 5 minutes each rinse. All sections were then stored in 0.1 M PB at 4°C for up to 24 hours until mounting. Sections were mounted onto FisherBrand Plus slides and then cover-slipped with DePeX mounting medium (Sigma-Aldrich, St. Louis, MO).

**Photos and image analysis**

Images were taken of brain sections under a 10× objective microscope at a magnification ×100 and a digital camera. Localization of specific brain regions was determined using the Allen Mouse Brain Atlas (Lein et al., 2007). For each brain region, 2–3 brain sections per mouse were imaged. Images were then cropped to include only the exact portion of each brain region by overlaying images from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2004) over the photos in an image editing program. Particles were then analyzed with the batch function using a macro in ImageJ (Schneider, Rasband, & Eliceiri, 2012). Twenty-five...
separate brain regions were processed: bed nucleus of the stria terminalis (BNST), lateral septum (LS), anterior hypothalamus (AH), medial preoptic area (mPOA), ventromedial hypothalamus (VMH), medial amygdala (meA), dorsolateral periaqueductal grey (dlPAG), ventrolateral periaqueductal grey (vlPAG), dorsal and ventral premammillary nuclei (PMd and PMv), cingulate cortex, infralimbic and prelimbic regions of the prefrontal cortex (IL, PrL), piriform cortex, retrosplenial cortex (RC), area CA1 of the hippocampus (CA1), area CA3 of the hippocampus (CA3), the dentate gyrus (DG), anterior cortical amygdala (ACA), central amygdala (CeA), basolateral amygdala (BLA), arcuate nucleus (Arc), lateral hypothalamus (LH), primary auditory cortex, primary visual cortex. All subjects for each brain region were analyzed concurrently, with each brain region being analyzed separately.

**Statistical analysis**

All statistical analyses were undertaken in R version 3.4.0 (R Core Team, 2016) in RStudio version 1.0.143 (RStudio Team, 2015).

**Behavioral analysis**

For each cohort, the linearity of the social hierarchy was calculated using Landau’s Modified $h'$. Briefly, the total number of wins by each individual against all other individuals are entered into a sociomatrix. Landau’s method then assesses the degree to which each individual consistently dominates others in contests and whether individuals can be linearly ordered based upon their wins and losses. The $h'$ value ranges from 0 (no linearity) to 1 (completely linear). The significance of $h'$ is determined by performing 10,000 two-step Monte Carlo randomizations of the sociomatrix and comparing the observed $h'$ against a simulated distribution of $h'$ (De Vries, 1995; Williamson, Lee, et al., 2016). Temporal changes in individual dominance ratings were calculated using Glicko Ratings (Glickman, 1999; So et al., 2015). Glicko ratings are a pairwise-contest model ratings system where ratings points are recalculated following each successive win or loss. All individuals start with a rating of 2200. Ratings are gained after wins and lost after losses with the magnitude of points gained or lost dependent upon the difference in ratings scores between the two individuals in each contest (Glickman, 1999; Williamson, Lee, et al., 2016). Landau’s modified $h'$ was calculated.
using the R package compete v0.1 (Curley, 2016a). Glicko ratings were calculated using the PlayerRatings package v1.0 in R (Stephenson & Sonas, 2012).

**Social ascent analysis**
To compare wins and losses between betas in the alpha removed group to those in the alpha remained group, we used Wilcoxon rank sum tests. To compare wins and losses between betas in the alpha removed and sham-removed group on the day of removal or sham-removal to their behavior the day before, we used Wilcoxon signed rank tests.

**Fos analysis**
To determine the effect of alpha removal on the number of immunoreactive cells in each brain region, we used the R package lme4 (Bates et al., 2015) to run negative binomial mixed models, with social status (alpha removed or alpha remained) as a fixed effect and cohort, removal number, side of the brain, number of wins, and number of losses as random effects. This model was run separately for each of the 25 brain regions. We chose $p = 0.01$ as our alpha level in order to decrease the chance of type 1 error without inflating type 2 error.

**Hierarchical clustering analysis**
To determine brain region activation patterns in both the alpha removed and alpha remained groups, we created correlation matrices for each group and visualized them using the R package lattice (Sarkar, 2017). We then used the package pvclust (Suzuki & Simodaira, 2015) to determine hierarchical clusters and generate p-values for each cluster using multiscale bootstrap resampling.

**Results**

**All cohorts form significantly linear hierarchies**
All social groups formed significantly linear dominance hierarchies with a clear alpha and beta male after the first four days of group housing prior to the first alpha or sham-removal (all $h’$ values $>0.45$, mean $h’ = 0.59$, all $p < 0.05$, mean $p = 0.016$). All alpha males maintained their alpha status for the duration of their presence in the established social hierarchy.

**Subdominant males socially ascend following removal of the dominant male**
After each of the 12 alpha male removals, a subdominant male ascended within 1 hour. Rising subdominants had significantly more wins than the subdominant males in the sham-removal group (Wilcoxon rank sum test $W = 138$, $p = 0.00018$ – Figure 2(a)). Further, the majority (9/12) of rising subdominant individuals never lost a fight during this period, however there was no significant difference in number of losses between rising subdominants in the alpha removed group and those in the sham removal group (Wilcoxon rank sum test $W = 56.5$, $p = 0.3006$ – Figure 2(b)). When compared to their behavior the day before alpha removal, rising subdominants had significantly more wins (Wilcoxon signed rank test $V = 1$, $p = 0.003$) and significantly fewer losses (Wilcoxon signed rank test $V = 48$, $p = 0.037$). There was no significant difference in wins (Wilcoxon signed rank test $V = 21$, $p = 0.54$) or losses (Wilcoxon signed rank test $V = 16$, $p = 0.832$) in non-rising subdominants on the day of sham removal when compared to the day before alpha removal (Figure 2(c)). In the alpha removed group, latency to first win occurred on average at 14.9 minutes, with some individuals winning their first fight within 15 seconds. This was significantly different from in the sham removal group, where latency to first win occurred on average at 34.9 minutes (Wilcoxon rank sum test: $W = 36.5$, $p = 0.042$; Figure 2(d)).

In 11/12 of the removals, the rising subdominant was predicted based on data from the previous three days prior to the alpha removal. In these 11 cases, the rising subdominant was the male with the second highest Glicko ranking (i.e., the beta male) prior to removal. In the one instance where this was not the case, the individual that ascended had a slightly lower Glicko ranking than the previous beta male. However, it is worth noting that this was in the sixth removal after many manipulations of the social group, and the alpha male in this group was extremely despotic performing over 80% of all aggressive acts within the group. Consequently, fewer social contests occurred between lower-ranked individuals making it difficult to unequivocally identify the ranks of all other lower-ranked males at this time-point.

**Social ascent is associated with differential Fos immunoreactivity throughout the brain**
Table 1 describes Fos immunoreactivity pattern for beta males in the alpha removed and alpha remained conditions for each brain region studied. For 15/25 brain regions, there was a significant difference in Fos immunoreactivity, with individuals from the alpha removed group displaying significantly higher numbers of immunoreactive cells (Table 1). Consistent with our predictions, 5 of these regions (BNST, LS, AH, mPOA, dlPAG) are areas within the Social Behavior Network.
The remaining 9 regions included prefrontal cortex (cingulate, infralimbic, prelimbic) as well as the retrosplenial cortex, hippocampal regions (CA1 and dentate gyrus), and a hypothalamic region (arcuate nucleus). Both the auditory cortex and visual cortex displayed increased immunoreactivity in the alpha removed condition. See Figure 3 for sample images of Fos staining.

Hierarchical clustering analysis suggests differential patterns of activation in individuals undergoing social ascent

To examine whether social ascent lead to differential co-activation patterns throughout the brain, we performed a hierarchical clustering analysis. Significantly different clusters between individuals undergoing social ascent and those in stable social groups were identified. In the alpha removed group, two distinct clusters formed, one including IL, PrL, DG, LS, CA3, vIPAG, LH, meA, Cing, RC, BLA, BNST, and CA1 and one including Aud, AH, PMv, CeA, Vis, CortA, dIPAG, mPOA, Pir, PMd, ARC, and VMH (Figure 4(a)). In the alpha remained group one cluster contained all regions but the BLA, IL, and PrL, with the IL and PrL splitting off into their own cluster (Figure 4(b)). Notably, in the alpha removed group we saw greater positive correlation between brain regions (Figure 4(c)) and in the alpha remained group, we saw greater negative correlation between brain regions (Figure 4(d)).
### Table 1. Table of Fos cell counts and results of mixed models for all brain regions examined.

<table>
<thead>
<tr>
<th>Region</th>
<th>Network</th>
<th>Bregma (mm)</th>
<th>Alpha Removed Mean ± SEM</th>
<th>Alpha Remained Mean ± SEM</th>
<th>β ± SEM</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNST</td>
<td>SBN</td>
<td>0.38–0.14</td>
<td>44.6 ± 11.3</td>
<td>21.8 ± 4.9</td>
<td>1.1 ± 0.4</td>
<td>0.008</td>
</tr>
<tr>
<td>LS</td>
<td>SBN</td>
<td>0.38–0.14</td>
<td>96.0 ± 27.8</td>
<td>19.7 ± 4.2</td>
<td>1.9 ± 0.7</td>
<td>0.004</td>
</tr>
<tr>
<td>AH</td>
<td>SBN/social def.</td>
<td>−0.46 to −0.58</td>
<td>69.0 ± 12.2</td>
<td>39.3 ± 7.4</td>
<td>0.5 ± 0.2</td>
<td>0.004</td>
</tr>
<tr>
<td>mPOA</td>
<td>SBN/social def.</td>
<td>0.14 to −0.10</td>
<td>91.5 ± 12.3</td>
<td>66.5 ± 14.4</td>
<td>1.1 ± 0.1</td>
<td>2.0E-016</td>
</tr>
<tr>
<td>VMH</td>
<td>SBN/social def.</td>
<td>−1.46 to −1.70</td>
<td>19.5 ± 4.8</td>
<td>8.2 ± 2.5</td>
<td>0.3 ± 0.4</td>
<td>0.492</td>
</tr>
<tr>
<td>meA</td>
<td>SBN/social def.</td>
<td>−1.06 to −1.22</td>
<td>67.9 ± 16.9</td>
<td>27.5 ± 5.3</td>
<td>0.2 ± 0.4</td>
<td>0.654</td>
</tr>
<tr>
<td>dIPAG</td>
<td>SBN/social def.</td>
<td>−2.92 to −3.16</td>
<td>20.6 ± 3.5</td>
<td>12.5 ± 2.2</td>
<td>1.6 ± 0.3</td>
<td>6.23E-06</td>
</tr>
<tr>
<td>vIPAG</td>
<td>SBN/social def.</td>
<td>−2.92 to −3.16</td>
<td>12.7 ± 3.2</td>
<td>6.3 ± 1.4</td>
<td>1.0 ± 0.4</td>
<td>0.023</td>
</tr>
<tr>
<td>PMd</td>
<td>social def.</td>
<td>−2.70 to −2.92</td>
<td>24.2 ± 3.6</td>
<td>19.2 ± 3.1</td>
<td>0.3 ± 0.2</td>
<td>0.100</td>
</tr>
<tr>
<td>PMv</td>
<td>social def.</td>
<td>−2.70 to −2.92</td>
<td>37.5 ± 5.0</td>
<td>35.9 ± 5.9</td>
<td>0.3 ± 0.2</td>
<td>0.159</td>
</tr>
<tr>
<td>Cingulate</td>
<td>Cortical</td>
<td>2.34–2.10</td>
<td>48.8 ± 14.8</td>
<td>10.2 ± 1.8</td>
<td>1.6 ± 0.3</td>
<td>2.67E-06</td>
</tr>
<tr>
<td>IL</td>
<td>Cortical</td>
<td>1.94–1.54</td>
<td>90.4 ± 14.0</td>
<td>12.9 ± 2.3</td>
<td>1.5 ± 0.3</td>
<td>5.49E-07</td>
</tr>
<tr>
<td>PrL</td>
<td>Cortical</td>
<td>1.94–1.54</td>
<td>142.2 ± 24.9</td>
<td>17.9 ± 3.4</td>
<td>2.0 ± 0.3</td>
<td>1.33E-11</td>
</tr>
<tr>
<td>Piriform</td>
<td>Cortical</td>
<td>1.94–0.02</td>
<td>198.5 ± 30.8</td>
<td>81.3 ± 18.1</td>
<td>0.6 ± 0.4</td>
<td>0.111</td>
</tr>
<tr>
<td>RC</td>
<td>Cortical</td>
<td>−0.94 to −1.94</td>
<td>34.7 ± 11.7</td>
<td>5.2 ± 1.6</td>
<td>1.4 ± 0.5</td>
<td>0.004</td>
</tr>
<tr>
<td>CA1</td>
<td>Limbic</td>
<td>−1.94 to −2.18</td>
<td>98.0 ± 22.1</td>
<td>17.6 ± 5.1</td>
<td>1.9 ± 0.4</td>
<td>3.32E-06</td>
</tr>
<tr>
<td>CA3</td>
<td>Limbic</td>
<td>−1.06 to 1.22</td>
<td>125.9 ± 28.3</td>
<td>61.6 ± 14.7</td>
<td>0.6 ± 0.3</td>
<td>0.005</td>
</tr>
<tr>
<td>DG</td>
<td>Limbic</td>
<td>−1.94 to −2.18</td>
<td>76.3 ± 12.6</td>
<td>25.1 ± 4.2</td>
<td>1.0 ± 0.3</td>
<td>0.003</td>
</tr>
<tr>
<td>ACA</td>
<td>Limbic</td>
<td>−0.46 to −0.82</td>
<td>44.5 ± 11.8</td>
<td>24.9 ± 7.4</td>
<td>0.6 ± 0.4</td>
<td>0.169</td>
</tr>
<tr>
<td>ceA</td>
<td>Limbic</td>
<td>−1.06 to −1.34</td>
<td>165.5 ± 14.5</td>
<td>145.6 ± 3.4</td>
<td>0.1 ± 0.7</td>
<td>0.730</td>
</tr>
<tr>
<td>BLA</td>
<td>Limbic</td>
<td>−1.06 to −1.34</td>
<td>91.3 ± 29.7</td>
<td>63.4 ± 17.8</td>
<td>1.1 ± 0.6</td>
<td>0.068</td>
</tr>
<tr>
<td>Arc</td>
<td>Limbic</td>
<td>−1.46 to −1.70</td>
<td>37.1 ± 10.8</td>
<td>10.8 ± 2.1</td>
<td>2.0 ± 0.3</td>
<td>3.78E-09</td>
</tr>
<tr>
<td>LH</td>
<td>Limbic</td>
<td>−0.46 to −0.58</td>
<td>31.6 ± 6.2</td>
<td>19.5 ± 2.4</td>
<td>0.5 ± 0.3</td>
<td>0.062</td>
</tr>
<tr>
<td>Auditory</td>
<td>Sensory</td>
<td>−2.18 to −2.80</td>
<td>120.2 ± 44.3</td>
<td>18.7 ± 4.1</td>
<td>2.1 ± 0.6</td>
<td>4.60E-04</td>
</tr>
<tr>
<td>Visual</td>
<td>Sensory</td>
<td>−2.54 to −2.92</td>
<td>143.4 ± 34.6</td>
<td>17.4 ± 4.0</td>
<td>1.7 ± 0.4</td>
<td>3.05E-05</td>
</tr>
</tbody>
</table>

### Figure 3. Sample images of Fos staining in individuals from the alpha remained (left) and alpha removed (right) groups.

(a) Prelimbic and infralimbic regions of the medial prefrontal cortex. The dotted line indicates the separation between the IL and PrL regions. (b) CA1 region of the hippocampus. Dotted lines indicate the edges of CA1. (c) Medial preoptic area of the hypothalamus. Lines indicate the edges of the mPOA.
suggesting that there was generally more coordinated activation of pathways in the individuals undergoing social ascent.

**Discussion**

In the present study, we successfully replicated the behavioral findings from our previous work (Williamson, Romeo, et al., 2017), illustrating that following removal of the alpha male, beta males recognized the emergence of a power vacuum and use this opportunity to ascend to alpha status. Ascending males won significantly more and lost significantly less in comparison to their own behavior the day before, as well as compared to non-ascending beta males in hierarchies whose alpha had not been removed. Moreover, this change occurs rapidly with beta males beginning their ascent on average within 15 minutes. These findings provide further evidence that individuals in social groups recognize and behaviorally respond to dynamic changes in social context. This ability appears to be a fundamental feature of living within a social group, and has been seen to occur in a similarly controlled manner in African cichlid fish (Maruska & Fernald, 2010; Maruska et al., 2013) where beta males begin to change color and increase aggression in response to alpha removal within minutes, and in primates, where beta males quickly and forcefully ascended to alpha status following alpha males receiving amygdaloid lesions (Rosvold, Enger, Mirsky, & Pribram, 1954). This ability for individuals to recognize and rapidly respond to changes in social status is an essential feature of social competence and is associated with greater social, reproductive, and health outcomes (Hofmann et al., 2014; Taborsky & Oliveira, 2012).

In the current study, we also demonstrate that response to a change in social context is associated increases in neural activity. Notable increases in immediate early immunoreactivity were observed throughout the SBN as well as in the prefrontal cortex and hippocampus. It appears that coordinated activation of these regions is required to facilitate the assessment of a change in the social context combined with the increase in aggressive behavior to facilitate social ascent. In the SBN, we saw significant differences in cell counts between ascending beta males and stable beta males in the BNST, lateral septum, mPOA, anterior hypothalamus, and the dIPAG. Each of these regions has well-established roles in the modulation of social behavior (Goodson, 2005). Significantly, we did not see

**Figure 4.** Beta individuals undergoing social ascent have distinct neural activation patterns as compared to beta individuals from stable groups. (a, b) Cluster dendrogram displaying results of hierarchical clustering analysis for beta males where the alpha was removed (a) and for beta males where the alpha remained (b). Red numbers indicate the approximately unbiased p-value generated through multiscale bootstrap resampling. Values higher than 95 indicate statistical significance. Red boxes denote significant clusters that are strongly supported by data. (c, d) Pearson correlation coefficients were used to create a heatmap of neural co-activation across examined brain regions. (c) Heatmap for beta males where the alpha was removed (d) Heatmap for beta males where the alpha remained.
any difference in neural activity in the VMH or medial amygdala. The VMH has been demonstrated to be of particular interest in female social behavior (Goodson, 2005), for example when females are assessing the social dominance of potential matres (Desjardins, Klausner, & Fernald, 2010) or in female aggression (Hashikawa et al., 2017). Further, the VMH has been shown to be involved in response to territorial challenge and stress (Goodson & Evans, 2004) but does not appear to be involved in the processing of more general changes in social context. The lack of difference in the medial amygdala is more remarkable, as it has been heavily implicated in social dominance (Bolhuis, Fitzgerald, Dijk, & Koolhaas, 1984; Rosvold et al., 1954; So et al., 2015; Timmer, Cordero, Sevelinges, & Sandi, 2011). However, these previous findings appear to be specific to stable social groups and to understanding the physiology of individuals of dominant vs. subordinate status and cannot be assumed to extend to individuals responding to a changing social context and subsequently undergoing a change in social status.

The largest fos immunoreactivity differences observed between ascending beta males and non-ascending beta males were in the prelimbic and infralimbic regions of the prefrontal cortex. In non-ascending beta males we find very little activation in these regions, whereas in ascending males we find very large levels of activation. These regions of the medial prefrontal cortex have been established as essential to the processing of rodent social behavior, including aggressive behavior, affiliative behaviors, and dominance behavior (Ko, 2017; Wang, Kessels, & Hu, 2014). In mice, prelimbic neurons have also been implicated in processing social preference as well as social-spatial information (Murugan et al., 2017). Further, the mPFC has been implicated in humans in the processing of social status information (Silk et al., 2017; Wang et al., 2014; Zerubavel et al., 2015) and the social network position of others (Parkinson, Kleinbaum, & Wheatley, 2017) as well as processing information in relation to self—i.e., how one fits into the broader social context (Pfeifer et al., 2009). Other studies have shown activation of the mPFC when processing unstable social hierarchies (Zink et al., 2008). In pairs of rhesus monkeys, the dominant individual’s PFC becomes locked in an “up-state” while the subordinate individual’s becomes locked in a “down-state”. This state rapidly switches when relative hierarchical status is switched (Fujii, Itohara, & Tonegawa, 2000). Neurons in the ventral hippocampus are necessary for social memory storage and are specifically activated in response to familiar mice (Okuyama, Kitamura, Roy, Iltohara, & Tonegawa, 2016). Before a beta individual begins his ascent, we observe patrolling and olfactory exploration of the vivarium by these males. During this exploration, the beta male is coming into contact with the urine of the familiar alpha male, a highly salient signal of social status (Lee, Khan, & Curley, 2017). This exploration could potentially lead to activation of the CA1 in response to social memory of interaction with the alpha. Notably, there is a clear, excitatory pathway from CA1 to the prelimbic region of the prefrontal cortex (Thierry, Gioanni, Dégénétais, & Glowinski, 2000), suggesting that the processing of social memory information in CA1 could be integrated with social context information being processed in PrL through this excitatory pathway. However, additional studies are required to elucidate the exact activation patterns connecting these brain regions. Further, both the dentate gyrus and the retrosplenial cortex have been implicated in regulating spatial memory (Czajkowski et al., 2014; Ibi et al., 2008; Jessberger et al., 2009; Nilsson, Perflilieva, Johansson, Orwar, & Eriksson, 1999; Ophir, Wolff, & Phelps, 2008). In a changing complex social environment, determining the physical presence or absence of more dominant individuals is critical and the observed activation of these brain regions in ascending males may be related to utilization of spatial memory. Moreover, oxytocin receptor signaling in the dentate gyrus has also been shown to be necessary for discrimination of social stimuli (Raam, McAvoy, Besnard, Veenema, & Sahay, 2017), providing further evidence for the importance of this brain region in processing social contextual cues.

We observed significantly elevated activation in the primary visual cortex and primary auditory cortex of ascending males. While studies of mouse social behavior often do not focus on the primary visual cortex, it is essential to social processing in humans—social visual
signals provide information about emotional expression, direction of gaze, body posture, and movement, all important social cues (Adolphs, 2003). Studies in non-human primates have demonstrated that neuronal responses in the visual cortex appear to encode highly specific social stimuli such as those described above (i.e., faces, gaze, etc.) (Perrett, Rolls, & Caan, 1982). While there is limited work to suggest that processing of visual stimuli is essential to mouse social behavior, studies of mouse models of autism suggest that excitatory/inhibitory balance and plasticity in the visual cortex during critical periods in development is important for the development of social behavior (Gogolla et al., 2009). Further, lack of proper gamma oscillations generated in the primary visual cortex are similarly implicated in autism, and have been shown to be important for information processing and learning, suggesting that these oscillations are important for appropriate social behavior (Gogolla et al., 2009; Singer, 1993). It is likely that the activation of the visual cortex during social opportunity is related to visual monitoring of the social environment. The impact of changing social context on the primary auditory cortex is consistent with the established role of auditory cues in communication in mice. Ultrasonic vocalizations (USVs) have been demonstrated to facilitate social interactions in mice (Liu, Miller, Merzenich, & Schreiner, 2003). Dominant males have been shown to elicit significantly more of these vocalizations in mating situations (Lumley, Sipos, Charles, Charles, & Meyerhoff, 1999; Nyby, Dizinno, & Whitney, 1976), though not necessarily during aggressive encounters (Nyby & Whitney, 1978; Portfors, 2007). These USVs may function as territorial signals between males mice (Gourbal, Barthelemy, Petit, & Gabrion, 2004; Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2012). These findings lead us to hypothesize that alpha males most likely emit USVs on a regular basis in the vivarium and that subordinates process the auditory inputs from the environment to determine if the alpha male is present or absent. Our analyses of immediate early gene activation indicated different co-activation patterns in the beta males undergoing social ascent from those in a stable social group. Most notably, those in the alpha removed group had overall increased, positively correlated activation throughout the regions studied, whereas those in the alpha remained group showed a more negative correlation throughout these regions. Specifically, we show through hierarchical clustering analysis that individuals in the alpha removed group have specific regional clusters of co-activation. The first cluster contains several brain regions (IL, PL, cingulate and retrosplenial cortices, CA1, CA3 and dentate gyrus) that are recognized as being critically important for the retrieval of social, emotional and spatial memories as well as the monitoring of contextual information and prediction of future events (Bicks, Koike, Akbarian, & Morishita, 2015; Eichenbaum, 2017; Tovote, Fadok, & Lüthi, 2015). It is conceivable that this cluster’s co-activation occurs following the removal of the alpha male from the social group when beta males recognize the changes in social and spatial contexts occurring within their environment. We propose that the coordinated patterns of activation identified in the second cluster may be related to the output of behavior. This cluster includes hypothalamic and midbrain regions of the SBN including the AH and mPOA. The increased frequency of aggressive behavior as well as increased activity such as patrolling behavior may be associated with the increased activation throughout these areas. Clearly, successful social ascent requires the integration of activation in those regions in cluster 1 that are associated with social cognition and those nodes of the SBN in cluster 2 that promote behavioral output. Although we do not know where this integration occurs, it is notable that two nodes of the SBN, the BNST and LS, both showed increased levels of Fos immunoreactivity in socially ascending males and clustered with cluster 1. These more anterior brain regions along with the MeA (which also clustered with cluster 1 but did not show a significant difference between groups) are known to be key relays between hypothalamic and midbrain regions of the SBN and other brain regions that comprise a social-decision making network including the hippocampus and frontal cortex (O’Connell & Hofmann, 2011). Taken together with our data, we hypothesize that the BNST and LS integrate intrinsic and environmental cues in response to the removal of the alpha male from the social group to produce the contextually appropriate behavioral responses of increased aggression in beta males. Future research will mechanistically address the biological significance of each of these regions in facilitating social ascent of beta males during social opportunities.

**Conclusion**

In the present study, we established that following removal of the alpha male from a stable dominance hierarchy, the beta male recognizes the absence of the alpha and responds by rapidly increasing aggressive behaviors. We have demonstrated that this salient social stimulus of removing the alpha male and subsequent change in behavior by the beta male leads to increased Fos immunoreactivity throughout the brain, specifically in regions of the SBN, as well as the medial prefrontal cortex, retrosplenial cortex and area CA1 of the hippocampus. These findings suggest that the complex social competence required to assess one’s social context and respond appropriately is modulated by a
synchronous and integrated increase in activity throughout the brain.

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