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Final Report

‘QUANTITATIVE ANALYSIS OF THE DYNAMICS OF JNK ACTIVATION IN THE EMBRYONIC CNS OF *DROSOPHILA MELANOGASTER*’

Introduction:

My work aims to elucidate the role of JNK signaling pathway during the embryonic Central Nervous System development (CNS) of *Drosophila melanogaster*.

CNS development consists of a series of strictly timed cellular events, initiated by neuroblasts specification and delamination, in the early *Drosophila* embryo. Subsequent events include the differentiation of neurons and glia, as well as the directed migration of axonal growth cones through the forming neuropile. In later stages, pattern refinement of the embryonic CNS relies on the programmed cell death of certain glial and neuronal populations [1].

JNK signaling has a well-established role in controlling differentiation, cell migration and apoptosis [2]. In *Drosophila*, JNK (*basket*) is the main mediator of morphogenetic apoptosis, while it has been shown to affect several collective cell movements during development [3; 4]. Remarkably, recent studies have indicated a role for Basket in controlling axonal stability and growth [5].

Further, JNK signaling, due to its key regulatory role on the aforementioned cellular functions, is subjected to tight regulation. In most *Drosophila* tissues Basket has been shown to be activated by the upstream MAPK-kinase Hemipterous (Hep), while the dual specificity phosphatase Puckered has been reported to specifically down-regulate the pathway [6]. Moreover, Puckered directly controls the activation levels of Basket through the formation of a negative feedback loop, since its expression depends on Basket activity. This negative feedback loop is essential for the dynamic modulation of the pathway outputs [6; 7].

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Aim of my visit to Dr. Martin-Blanco's laboratory:

The objective of my visit to Dr. Martin-Blanco's laboratory at the IBMB-CSIC in Barcelona – Spain was to use a recently developed by the host lab dJun-FRET biosensor in order to quantitatively analyze the dynamics of JNK activation in the developing embryonic CNS of *Drosophila melanogaster*. In particular and due to time limitations my interest focused in examining the functionality of the FRET-sensor as well as in monitoring the dynamics of JNK activation during the embryonic CNS development.

Results:

The dJun-FRET sensor is a unimolecular construct carrying a CFP-YFP pair of monomeric fluorophores separated by a modified JNK-substrate sequence (phosphorylation site of the transcription factor Jun) and a phospho-threonine binding domain. Phosphorylation of the substrate sequence by JNK results in an intramolecular clamp between the substrate sequence and the phospho-threonine domain thus inducing / increasing energy transfer from the CFP to the YFP [8]. This construct was placed under the control of the UAS-sequence in order to facilitate its GAL4-driven expression in different *Drosophila* tissues in transgenic flies.

For the calculation of FRET efficiency a ratiometric method was applied using the equation $E_{FRET} = I_{DA} / I_{DD}$, where E corresponds to FRET efficiency and I_{DA} and I_{DD} correspond to pixel intensity within a region of interest (ROI) upon acceptor emission during donor excitation and donor emission during donor excitation respectively (the protocol published by Kardash et al was adopted to our experimental needs) [9].

Preliminary results from our lab have shown that *puckered* gene function is necessary for the development of the embryonic nervous system and specifically for the proper fasciculation of the longitudinal axonal tracts. In addition, analysis of *puckered* reporter lines has revealed that Puckered is present in distinct neuronal populations but not in glial cells. Interestingly, *puckered* was expressed in subsets of pioneer motor neurons, such as the aCC and RP2 neurons.

Initially, and in order to examine the functionality of the dJun-FRET sensor, we targeted its expression in aCC/RP2 class of motor neurons.

Using the RN2-GAL4 and CQ-GAL4 lines, active in aCC/RP2, we expressed the UAS-dJun-FRET in the aforementioned motor neurons. The construct was successfully expressed although the intensity of the CFP/YFP signal was rather dim at early stages of CNS development (embryonic stages 12-15) increasing gradually towards the end of embryogenesis.

Employing the same GAL4 lines we coexpressed along with the dJun-FRET sensor either a UAS-HepCA or a UAS-Puc2A constructs. Over-expression of the constitutively active form of Hemipterous (UAS-HepCA) resulted in an expected overactivation of the JNK pathway, which let us to quantify the highest level of the FRET efficiency of the dJun-FRET sensor. In contrast, over-expression of Puckered (UAS-Puc2A) downregulated the pathway and the FRET efficiency in this condition was assigned to the minimum level of JNK activity.

Applying the parameters defined by the aforementioned control experiments we found that FRET is evident in the cell body of aCC/RP2 neurons in the later stages of embryogenesis, although we were unable to follow their development in living embryos.

Moreover, in order to validate the specificity of the sensor we expressed the UAS-dJun-FRET construct in all glia cells employing the Repo-GAL4. As mentioned above, the *puckered* gene is strictly expressed in neurons and is not detected in glial cells, implying low levels of JNK activation in this cell type. Indeed, the FRET efficiency of the sensor in glia, was similar to that calculated for Puckered overexpression conditions, thus being indicative of low JNK activity.

From the above experiments we conclude that the dJun-FRET biosensor is fully functional in the embryonic CNS and is a reliable tool for the analysis of JNK activation dynamics *in vivo*.

Next, we examined the activation of the sensor during the development of the embryonic CNS. For that purpose, we expressed the UAS-dJun-FRET construct in all post-mitotic neurons by means of the Elav-GAL4. In control experiments the UAS-HepCA and UAS-Puc2A constructs were co-expressed, as described above. In order to avoid developmental problems induced by the

expression of the HepCA and Puckered in the nervous system, we used the recombined version of the Elav-GAL4 driver with the GAL80 thermo-sensitive GAL4-suppressor and expression of the UAS constructs was induced for 3 hours at 29^{o}C, prior to imaging. In all cases imaging was initiated at stage 12 and terminated at late stage 16 due to the increasing contractions of the embryonic musculature, which severely affect confocal imaging.

From these experiments we observed that high JNK activity is evident in the soma of the pioneer midline neurons, as well as in the soma of a few (unidentified so far) neurons of the peripheral nervous system during the embryonic stages 12-14. In particular, in the CNS midline we observed a segmental pattern of JNK activity, where in each neuromere, only anterior cells were exhibiting high FRET efficiency. In stages 14-15, high JNK activity was also detected in macrophages, persisting until the end of embryogenesis. Surprisingly, minimum FRET was detected in axons of the central and peripheral nervous system, while moderate FRET efficiency was observed in the developing chordotonal organs. Finally, high JNK activation was observed during ventral nerve cord condensation at stage 15-16, when the neurons of the last two posterior abdominal segments undergo apoptosis.

Concluding remarks and considerations:

The processing of the acquired data during my stay in the host lab is not yet completed. The main reason for that is that image processing of time-lapse experiments and FRET efficiency calculations require a considerable amount of time. Currently, and in collaboration with the host laboratory we are trying to improve the processing protocol by building an ImageJ macro, to facilitate this step.

In addition, all initial experiments were performed in a standard confocal microscope, where the signal to noise ratio was not allowing us to obtain fully reliable data, probably because the expression of the sensor in the CNS was not satisfactory. In November 2012, the host lab obtained an improved confocal microscope carrying a full spectral photon counting detector, which made the performance of the experiments described here, feasible.

References:

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