Differential Evolutionary Wiring of the Tyrosine Kinase Btk

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Abstract

Background: A central question within biology is how intracellular signaling pathways are maintained throughout evolution. Btk29A is considered to be the fly-homolog of the mammalian Bruton’s tyrosine kinase (Btk), which is a non-receptor tyrosine-kinase of the Tec-family. In mammalian cells, there is a single transcript splice-form and the corresponding Btk-protein plays an important role for B-lymphocyte development with alterations within the human BTK gene causing the immunodeficiency disease X-linked agammaglobulinemia in man and a related disorder in mice. In contrast, the Drosophila Btk29A locus encodes two splice-variants, where the type 2-form is the more related to the mammalian Btk gene product displaying more than 80% homology. In Drosophila, Btk29A displays a dynamic pattern of expression through the embryonic to adult stages. Complete loss-of-function of both splice-forms is lethal, whereas selective absence of the type 2-form reduces the adult lifespan of the fly and causes developmental abnormalities in male genitalia.

Methodology/Principal Findings: Out of 7004–7979 transcripts expressed in the four sample groups, 5587 (70–79%) were found in all four tissues and strains. Here, we investigated the role of Btk29A type 2 on a transcriptomic level in larval CNS and adult heads. We used samples either selectively defective in Btk29A type 2 (Btk29A[strong]) or revertant flies with restored Btk29A type 2-function (Btk29A[weak], Exc1–16). The whole transcriptomic profile for the different sample groups revealed Gene Ontology patterns reflecting lifespan abnormalities in adult head neuronal tissue, but not in larvae.

Conclusions: In the Btk29A type 2-deficient strains there was no significant overlap between transcriptomic alterations in adult heads and larval neuronal tissue, respectively. Moreover, there was no significant overlap of the transcriptomic changes between flies and mammals, suggesting that the evolutionary conservation is confined to components of the proximal signaling, whereas the corresponding, downstream transcriptional regulation has been differentially wired.

Introduction

The evolution of gene expression is considered to mainly result from regulatory, rather than coding, mutations causing phenotypic differences [1]. Analyzing six different organs from ten different species it was recently reported that the rate of gene expression evolution varies among organs, lineages and chromosomes [2]. As gene products commonly function together in distinct combinations to fulfill specific tasks, concerted expression changes of selected genes may be relevant to the survival of the species. Along these lines of arguments, Brawand et al. described sets of different organ-specific modules, which were evolutionarily conserved [2].

In this study we have investigated the evolution of tyrosine kinase-based signaling, focusing on Bruton’s tyrosine kinase (BTK) in particular. While it is known that elements in proximal BTK-signaling are conserved even among distantly related species, it remains an open question as to whether this is also true for the entire pathways down to the effector level. Here we address this question at the transcriptomic level.

The sequence of Btk has been conserved throughout evolution, with an ancestor emerging already prior to the evolution of metazoans [3]. This kinase belongs to the Tec family of non-receptor tyrosine kinases (TFKs). While insects have only a single TFK, in vertebrates there are several kinase species, which have
evolved through gene duplications. The fly kinase is most homologous to vertebrate Btk. However, in spite of the high degree of sequence conservation, the functional role of Btk seems to vary throughout evolution. In higher organisms, such as humans and other mammals, the significance of Btk lies in its function for a normal development of the immune system [4].

In the absence of mammalian Btk, B-cell receptor signaling is insufficient for the generation of mature B-lymphocytes [5,6,7], resulting in the immunodeficiency disease X-linked agammaglobulinemia (XLA) in humans [8,9] and X-linked immunodeficiency disease (Xid) in mice [10,11]. Insects, like Drosophila, possess neither B- nor T-cells. An orthologous function for Drosophila Btk, i.e., regulating B-cell maturation, can therefore not be expected. The Drosophila Btk29A locus produces two different gene products, type 1 and type 2, respectively, by differential splicing. The type 2 form reveals the highest homology to human BTK among mammalian TKFs [12]. Thus, this variant is considered to be the fly homolog of Btk by means of protein sequence [13]. It is specifically required for longevity and for development of male genitalia in the fly [12].

The type 1 splice variant is shorter at the N-terminus and is unique to flies [3,12]. The Drosophila Btk29A locus displays a dynamic pattern of expression through the embryonic to adult stages [FlyAtlas. http://130.209.54.32/atlas/atlas.cgi [14]. The Btk29A^P^ is a unique allele in that it is devoid of transcription of the type 2 isoform, while leaving the type 1 isoform intact. Btk29A types 1 and 2 are both expressed in the central nervous system (CNS) and in the imaginal discs [12], which are epidermal thickenings in the larvae containing ecto- and mesodermal cells, which give rise to the adult organs during metamorphosis. Complete loss of function of the gene (i.e., loss of both types 1 and 2) in female germline cells, produced by using the dominant-female-sterile, FLP/FRT technique, results in oocyte undergrowth and subsequent embryonic death accompanied by defective head involution [15,16,17,18,19]. Offspring with selective loss of the type 2 transcript are viable, developing malformed male genitalia and a reduced adult life span [12]. Thus, the Btk29A locus exerts pleiotropic functions both through distinct spatio-temporal rining of expression as well as the generation of distinct forms of protein products by alternative splicing in various tissues. When Btk29A function is lost in a Src64 mutant background, cellularization becomes incomplete in the blastoderm-stage embryo [20] and late-staged embryos fail to complete dorsal closure [21]. In Btk29A mutant females, oogenesis is underdeveloped presumably due to deficits in the formation of ring canals that transfer cytoplasm from nurse cells to oocytes [15,16,22]. Both the cellularization and oocyte phenotypes appear to result from failure to activate actin–myosin contractions [20]. Chandrasekaran and Beckendorf et al. have shown that Btk29A controls both the actin cytoskeleton and the cell cycle in the morphogenesis of embryonic salivary glands [23]. Interactions between mammalian Btk and actin have also been reported in several settings [24,25,26,27,28], suggesting this to be a common denominator in the proximal part of the Btk-signaling pathway, i.e., proximal of the Btk-dependent transcriptional regulation.

In the present study we adopted a genome-wide approach to identify Btk-dependent targets in neuronal tissues by exploring the transcriptional output from Btk-deficient and wild-type tissues, for two developmental stages in Drosophila melanogaster, respectively. Genes identified in this way could be direct or indirect targets for Btk-regulated transcription and outline part of the transcriptomic role of Btk in the development of the fly. The identification of Btk targets, corroborated by statistical analyses and gene set enrichment analyses, reveals parts of the scope and complexity, which Btk plays in the fly. We also conclude that there is no significant functional transcriptomic conservation for Btk targets between mouse B-cells and neuronal tissue from Drosophila.

Results and Discussion

We performed transcriptional profiling of the central nervous system (CNS) tissues from mutant (Btk29A^+/^) and revertant (Btk29A^+/^ excL-16) adults and larvae using the Affymetrix Drosophila Genome 2 chips, with 18,880 probe sets covering around 13,500 genes. This analysis yielded a list of affected genes known to function in longevity and aging, two biological processes impaired in Btk29A^+/^ mutants, thus validating the experimental rationale and setup. Although the phenotype-genotype association in the
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A

Larvae CNS  Adult head

B

I. (40%)
II. (19%)
III. (16%)

Larvae CNS  Adult head

C

Correlation (R²)

-1.00  -0.60  -0.20  0.20  0.60  1.00

FicP L2  FicP L1  FicP L3  Rev L1  Rev L2  Rev L3  FicP H2  FicP H1  FicP H3

Revertant Btk29A(fic Exc1-16)
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Drosophila Btk29A locus has been studied in some detail, the components and regulators of Btk29A signaling remain unexplored on the global transcriptomic level. Also, the biological processes triggered by Btk signaling defects in Drosophila are less well understood. Moreover, to our knowledge, the comparison of changes in gene expression profiles between Btk mutants of different animal species has not been performed before. Here, we use the strength of the Drosophila system in order to identify candidate effectors that take part in the Btk signaling process and use these data to perform an inter-species comparison of Btk-dependent components between mouse and fruit fly. To achieve this we have made use of the Btk29A type 2 mutant (Btk29A 

Reversion

Verifying P-element Insertion Point and the Nature of the Reversion

In order to define molecularly the experimental flies, we initially determined the exact location of the P-element insertion. For this we made use of P-element-specific primers (directed outwards from both the 5’ and 3’-ends of the P-element) for Btk29A 

Transcriptional Profiling

The transcriptional profiles were analyzed with Affymetrix whole genome arrays (GeneChip Drosophila Genome 2.0) by a comparative approach between the “mutant” and “revertant” sample groups for either larvae or adult heads. Thus, in total, 4 different sample groups were collected (Figure 2A–C). To reconstruct strain and tissue trends in a global transcriptomic detail, we built an expression distance matrix for the four sample groups with its replicas and reconstructed a gene expression tree (Figure 2A). The tree is highly consistent with the expectation that...
the predominant factor to characterize the profile is the tissue type/developmental stage followed by the Btk-genotype. A quality measure for the data input is that the majority of replicates fall within the respective sample group (Figure 2A–C). To obtain an initial overview of the transcriptional expression patterns, we performed a principal-component analysis, which clearly separates the data according to sample group (Figure 2B). Figure 2C represents a Pearson correlation ($R^2$) matrix for the whole transcriptomic profile for all samples included in the study. Here we see a higher intra-tissue correlation between revertant and Btk29AficP mutant of the same developmental stage compared to the intra-strain correlation between the two stages/tissues (Figure 2C).

Under such circumstances, several mathematical approaches are possible in extracting the genes that behave differently according to sample groups. When applying an ANOVA filtering in the comparison between the mutant (Btk29AficP) and revertant (Btk29AExc1–16) results, irrespective of the stage/tissue (larvae CNS or adult head) using a $p$-value of 0.05, we found 523 transcripts being statistically different between the Btk29AficP and the revertant. On the other hand, when considering the stage/tissue as the decisive factor (regardless of using Btk29AficP or revertant data) we detected 4489 transcripts being statistically different between the groups. This indicates, as expected, that the difference between tissue types or developmental stages in the fly gives a stronger influence on the transcriptome compared to the influence of the Btk29AficP mutation. On the other hand, when both the tissue type (larvae CNS and adult head) and genotype (Btk29AficP and revertant) are considered as the decisive ANOVA factors with a $p$-value $<0.05$ we find 391 transcripts being statistically different between the 4 sample groups. Thus, on a transcriptomic level there are 391 transcripts that, by these criteria, are Btk-dependent in Drosophila neuronal tissue development, from larvae to adult flies.

A differential fold-change cut-off of Signal Log Ratio (SLR) >1.2 (difference of means between FicP and revertant data) was applied for the selection of transcripts. The number of transcripts found to be differentially expressed between the two genotypes was more than 4 times higher in adult heads (744) as compared to

Figure 4. GSEA for the 391 Btk-dependent transcripts during Fly neuronal development: Enriched Biological Process clusters within the list of 391-transcripts (Btk-dependent transcripts during fly neuronal development). Figure 4 should be statistically interpreted as follows: The nodes, corresponding to different Gene Ontology clusters, are either not colored (white) i.e. not found with statistical power or colored in the scale yellow to orange, where yellow nodes are found with statistical significance after Bonferroni correction $p<0.05$ and orange colored nodes are found to be even more statistically significant after correction, with a $p<7\times10^{-6}$. The grey-zoned data highlights statistically enriched clusters of nodes (genes/transcripts), which all are represented under the manually designated heading e.g. ‘Gland development’ or ‘Cell death’. Due to space limitations in the main figure (Figure 4) we are not able to list the genes belonging to each grey-zone and cluster. This information is instead found in the Figure S1.
larval CNS (167; Figure 3). This suggests that there are more Btk-dependent transcripts in the head, perhaps also reflecting the fact that the head is not only composed of neuronal tissue. The distribution of up- and down-regulated transcripts was approximately 50% in adult heads. In the larval CNS sample group, the distribution of differentially expressed genes/transcripts was approximately 30% up-regulated versus 70% down-regulated, suggesting that transcriptional loss-of-function is the predominating feature in the larval CNS of Btk29A\(^{Pr}\) mutants.

**Clustering of Genes and Functional Enrichment**

By performing gene set enrichment analysis using Cytoscape and the plug-in BiNGO we identified different Gene Ontology (GO) clusters being enriched in the different data sets. For the 391 probe sets indicated to be Btk-dependent during fly neuronal development we found four major Gene Ontology clusters to be enriched within this list (Figure 4 and a more detailed view is found in Figure S1 where the corresponding genes are listed to respective statistically significant nodes). Due to space limitations in the figures we were not able to enlarge all the titles of the nodes within the figure. We have instead tried to find commonalities within the grey-zone and manually put a 'heading' for each of these zones in order to give the reader an overview of the result of the GSEA. In order to see the node titles (corresponding to Gene Ontology Biological Process names) the reader can zoom in on the figure and by this be able to read the text. Amongst these clusters representing Death, Cell development, Metabolic process and Gland development we find genes previously linked to Btk function and fly development, thus validating the approach of our study, but also genes not previously known to be associated with Btk in the fly. As such, gene set enrichment analysis suggests that the genes identified reflect a bona fide response of fly neuronal development to the loss of Btk.

In the larval CNS we find 167 Btk-dependent transcripts being differentially expressed with an SLR \(>\)1.2 between Btk29A\(^{Pr}\) and revertant flies. A Gene Set Enrichment Analysis on this set of transcripts reveals Gene Ontology terms which mirror undifferentiated progenitor cells for the future adult i.e., ‘imaginal’ cells (Figure 5 and a more detailed view is found in Figure S2 where the corresponding genes are listed and colored depending on the direction of the regulation to respective statistically significant nodes). Indeed, one of the processes showing the highest statistical significance after Bonferroni correction \(p<0.05\) and orange colored nodes are found to be even more statistically significant after correction, with a \(p<7\times10^{-8}\). The grey-zoned data highlights statistically enriched clusters of nodes (genes/transcripts), which all are represented under the manually designated heading e.g. ‘Systems development’ or ‘Regulation of growth’. Due to space limitations in the main figure (Figure 5) we are not able to list the genes belonging to each grey-zone and cluster. This information is instead found in the Figure S2.

**Transcriptional Comparison between Mouse and Fruit fly Btk-defective Cells**

A central question in biology is to what level protein function in intracellular signaling pathways is conserved through evolution of species, like e.g. between mammals and insects. For instance, it has been proven possible to ‘humanize’ the fly by introducing human genes of interest, including the human BTK gene, and studying them in an organotypic context [13,29,30]. Previous studies on components in the JAK/STAT signaling pathway has revealed a small, but statistically significant, overlap between Drosophila and mammals at a transcriptomic level [31]. In sea urchins and sea stars, organisms that diverged from their common ancestor 500 million years ago, a three-gene feedback loop involving Notch-signaling controls endoderm and mesoderm development in both overlapping and distinct ways [32]. Furthermore, appendages of different insects show divergent use of developmental regulatory genes, including the helix-loop-helix, homeodomain transcription factor Distal-less [33]. These phenomena have been referred to as gene regulatory network ‘plug-ins’, in which sub-circuits are frequently re-deployed during evolution while the internal
structure remains the same [34]. Further example of such rewiring comes from protein kinase A (PKA) catalytic subunit signaling in the fungus of the genus, Cryptococcus [35]; two sibling species of this pathogen express two different catalytic subunits of PKA, and alternative subunits are used in virulence factor production and mating in each species. It is envisaged that an ancestral PKA underwent a duplication event leading to the two catalytic subunit genes, one of which retained its function for the given biological processes in each species. Whether the “unused” subunit has undergone neofunctionalization with a novel gain-of-function for another biological activity is not known, but this example demonstrates evolutionary reconfiguration of a signaling cascade.

**Figure 6.** GSEA for the 744 Btk-dependent transcripts found in adult head tissue: Enriched clusters of Biological Processes were found in the Btk-dependent Adult head tissue analysis and were statistically significant. Figure 6 should be statistically interpreted as follows: The nodes, corresponding to different Gene Ontology clusters, are either not colored (white) i.e. not found with statistical power or colored in the scale yellow to orange, where yellow nodes are found with statistical significance after Bonferroni correction p<0.05 and orange colored nodes are found to be even more statistically significant after correction, with a p<7*10^-6. The grey-zoned data highlights statistically enriched clusters of nodes (genes/transcripts), which all are represented under the manually designated heading e.g. ‘Transport’ or ‘Aging and life span’. Due to space limitations in the main figure (Figure 6) we are not able to list the genes belonging to each grey-zone and cluster. This information is instead found in the Figure S3.

| **Table 1.** 25 genes found to overlap between D. melanogaster larvae CNS and adult head. |
|---------------------------------------------------------------|
| **Gene symbol** | **FlyBase ID** | **Differentially regulated** | **Btk29A^MP** | **Btk29A^HP** |
|-----------------|----------------|-------------------------------------------------|----------------|----------------|
|                 |                | **Adult Head** | **Larva CNS**  |                |
| w               | FBgn0003996    | 4.37           | 3.49           |                |
| TnpC47D         | FBgn0010423    | 2.22           | 2.89           |                |
| A-Extl          | FBgn0015568    | 1.83           | 2.21           |                |
| (3)mbn          | FBgn0002440    | 1.39           | 2.18           |                |
| CG5597          | FBgn0034920    | 1.36           | 2.12           |                |
| CG5023          | FBgn0038774    | 1.28           | 1.62           |                |
| CG11807         | FBgn0033996    | 2.92           | 1.59           |                |
| CG4398          | FBgn0034126    | 1.91           | 1.53           |                |
| pnt             | FBgn0003118    | 1.4            | 1.31           |                |
| CG2177          | FBgn0039902    | 1.44           | 1.25           |                |
| mthIl3          | FBgn0028956    | −2.23          | −1.22          |                |
| pen-2           | FBgn0053198    | −1.84          | −1.25          |                |
| gdi-olf39       | FBgn0028377    | −1.23          | −1.4           |                |
| CG14033         | FBgn0046776    | −2.05          | −1.4           |                |
| pst             | FBgn0035770    | −1.59          | −1.56          |                |
| CG6984          | FBgn0034191    | −1.42          | −1.65          |                |
| CG11671         | FBgn0037562    | −2.01          | −2             |                |
| CG42254         | FBgn0259112    | −1.79          | −2.02          |                |
| CG17264         | FBgn0031490    | −1.72          | −2.07          |                |
| CG32368         | FBgn0052368    | −2.9           | −5.88          |                |
| CG12241         | FBgn0038304    | 1.46           | −1.29          |                |
| Dob             | FBgn0030607    | −1.87          | 1.61           |                |
| Obp56h          | FBgn0034475    | −3.52          | 2.12           |                |
| proPO-A1        | FBgn0261362    | −1.64          | 2.81           |                |
| CG11076         | FBgn0032682    | 1.22           | −1.28          |                |

*Italics denotes down-regulated genes.
*denotes genes differentially expressed NOT in the same regulatory direction for the larval CNS and adult head sample group.

Recently, rewiring of both prokaryotic and eukaryotic signaling pathways has been achieved using rational design, demonstrating another aspect of the alteration of signal transduction pathways [36,37].

Apart from the well-known developmental role in the immune system, mammalian Btk has been shown to exert two counteracting roles in apoptosis, one as a protector and in the other as an inducer of apoptosis depending on the context [38,39]. This reflects the diverse role of a protein within a species. In the fruit fly, loss of the Btk29A type 2 transcript is compatible with life, as opposed to loss of both types 1 and 2 of Btk29A, which is embryonic lethal. However, type 2 mutant flies display reduced life span as well as malformation of the male genitalia [15,16]. We have previously published work on Btk-defective mouse B-lymphocytes using gene expression profiling [40,41]. In order to identify factors with conserved functions throughout evolution we conducted an inter-species comparison of the Btk-dependent transcripts identified in Drosophila against our previous transcriptomic data obtained with mouse Btk-defective Transitional type 1 B-lymphocytes [40]. Figure 7 illustrates the number of transcripts found to be expressed in every Drosophila sample group examined and the level of overlap between the assemblies. This indicates that approximately 30% of the Drosophila genome is expressed at the time examined in the respective tissues and strains. In comparison to these numbers, in mammalian B-lymphocytes [40] we see that 37% of all transcripts in the mouse genome are expressed at any given time. Figure 8A shows a Venn-diagram that illustrates the overlap between the differentially expressed transcripts found in mouse Btk-defective Transitional type 1 B-cells (a total of 147 regulated genes) and the Drosophila Btk29A^HP adult head (a total of 744 differentially expressed transcripts). The overlap between the two species is only sixteen transcripts, corresponding to 13 genes in the Btk-defective mice found in our previous study [40] having orthologs in the Btk-dependent transcripts identified in Drosophila (Figure 8B). Of these 16 transcripts only 5 are found to show parallel changes in Drosophila and mice being either up- or down-regulated in the Btk-defective strains (denoted as * in Figure 8B). By analyzing the gene expression profile from Btk-defective flies representing two different developmental stages and comparing these to mammalian Btk-defective B-cells we conclude that there is no significant overlap in the transcriptome for Btk-defective mammalian B-cells and neuronal cells from Drosophila. Based on these observations, we conclude that there is no significant functional transcriptomic conservation for Btk targets between the mammals and fly species.

**Concluding Remarks**

Although the upstream signaling protein components of Btk29A seem to be conserved throughout evolution, the downstream transcriptional pattern seems not to be comparable between the fruit flies and mice. The Btk-dependent gene expression profile seen in mouse transitional type 1 B-lymphocytes from Btk-defective animals thus differs from the global transcriptomic signature seen in Btk29A type 2-defective neural tissues from Drosophila. This is in contrast to JAK/STAT signaling in which
Figure 7. Distribution of expressed transcripts in each sample group and their overlaps: Venn-diagram showing the number of expressed probe-sets of the respective sample group and their overlap. The probe-sets found to be expressed above background (Affymetrix P-calls) in all three replicates per sample group were considered within this figure.
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Figure 8. Comparison between Mouse and Drosophila Btk-dependent transcript: A) Venn-diagram: showing the overlap between Btk-dependent Transitional type 1 B-cells from Btk-defective mice [40] (a total of 147 differentially expressed genes) and the Btk-dependent transcripts found in Btk29AficP Drosophila adult head (a total of 744 differentially expressed genes). B) Thirteen orthologous transcripts found in Btk KO mouse Transitional type 1 B-lymphocytes and in Btk29A defective flies. Bar-graph showing the 16 transcripts, corresponding to 13 genes, found to be common in a homology search between the Btk-dependent transcripts found in the Btk KO mouse Transitional type 1 B-cells compared to its healthy control strain [40] and the current Drosophila Btk29AficP study. Out of these 13 genes, 5 genes showed the same regulatory direction i.e. being up- or down-regulated in the respective Btk-defective strain (denoted as *).
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both the upstream and downstream components were reported to be conserved [31].

Indeed, large scale profiling data must be interpreted with caution and the genes identified here await ultimate proof as to whether they represent the bona fide effectors of Btk29A-mediated developmental signaling. Although detailed mechanisms of action of individual effectors and their roles linked to Btk29A function remains partially unknown, it is interesting to note that a profile related to life-span was recognized, suggesting that our transcriptomical mapping approach has effectively identified different pathways and effectors likely to play roles in Btk signaling and functioning regarding fruit fly development.

Materials and Methods

Drosophila Melanogaster Strains

w:Btk29A\(^{d18P}\)/CyO and w1118; Btk29A\(^{die}\) Exc1–16/SMI were generated in the Yamamoto laboratory (http://www.jst.go.jp/erato/project/yks_P/yks_P.html). Flies were raised on standard medium on a 12:12 h L:D cycle, at 23°C and at 55% RH. The Btk29A\(^{d18P}\) chromosome was put over the CyO, P[w\(^{+\text{+MC]}\) = ActGFP]\(\text{JMR1}\) (source: Bloomington stock center) balancer using standard crossing schemes. This balancer was later used for sorting heterozygous (GFP-positive) from homozygous Btk29A\(^{d18P}\) mutants (GFP-negative). Btk29A\(^{die}\) Exc1–16 flies were kept homozygous viable in stocks.

P-element Breakpoint Determination using PCR

Four kb (2L:8274950,8279050) surrounding the Btk29A-locus was used as a template to construct 8 forward- and 8 reverse-oriented primers covering the entire 4-kb region from both ends with a 500 bp spacing. Primers were also made for the 3' and 5' ends of the Btk29A\(^{d18P}\)-responsible P-element (Bm\(^{\Delta w}\)). Both primers were facing outwards from the P-element. PCR was performed using ABI GeneAmp\textsuperscript{TM} system 2700 and the insertion site was determined by sequencing the PCR product (http://www.eurofinsdna.com).

Dissection and Sample Preparation

Flies were anaesthetized using CO\(_2\), then immediately dissected. The tissues dissected were the complete heads, severed at the neck from adult flies and the CNS (developing brain), including the optic lobes from third instar wandering stage larvae. Tissues were collected into RLT buffer, pooled and extracted for RNA using Qiagen RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA). RNA was collected into Agilent\textsuperscript{TM} Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), after RNA extraction and in vitro transcription steps. Drosophila genome 2 expression arrays were hybridized and read using standard Affymetrix procedures. Microarrays were run at the Bioinformatics and Expression Analysis core facility (http://apt.bea.ki.se/index.html) located at Karolinska Institutet, Huddinge (Novum).

RNA Isolation and Microarray Processing

RNA was extracted and in vitro reverse-transcribed according to Affymetrix protocol. Quality assurance was provided by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), after RNA extraction and in vitro transcription steps. Drosophila genome 2 expression arrays were hybridized and read using standard Affymetrix procedures. Microarrays were run at the Bioinformatics and Expression Analysis core facility (http://apt.bea.ki.se/index.html) located at Karolinska Institutet, Huddinge (Novum).

Gene Ontology (biological process) clusters for the 391 Btk-dependent transcripts found, compared to our previous transcriptomic data obtained from mouse Btk-defective Transitional type 1 B-lymphocytes [40], where a total of 147 Btk-dependent genes were reported as differentially expressed. The 147 genes found to be Btk-dependent in Btk-defective mouse Transitional type 1 B-cells were investigated for orthologs/homologs in the Drosophila melanogaster specie. We made use of the Affymetrix oligonucleotide array comparison to find which transcripts could be possible homologs (www.affymetrix.com). Both raw and pre-processed data was then scaled to a common median value. Both raw and pre-processed data was deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) (GSE30627).

Further filtering, within sample group analyses and pair-wise comparisons were carried out using dChip (https://sites.google.com/site/dchipsite/), http://biosun1.harvard.edu/complab/dchip/) and Microsoft Excel.

For the Venn-diagram (Figure 7), the probe-sets found to be expressed above background (Affymetrix P-calls) in all three replicates per sample group were considered within this figure.

Supporting Information

Figure S1 Gene Set Enrichment Analysis for Gene Ontology (biological process) clusters for the 391 Btk-dependent transcripts during Fly neuronal development (Figure 4 in manuscript): Enriched Biological Process clusters within the list of 391-transcripts (Btk-dependent transcripts during fly neuronal development). The genes belonging to respective cluster are written next to the grey-zoned areas. For the main Figure 4, Figure S1 shows the respective genes found for each cluster (grey-zoned in Figure 4).

Figure S2 Gene Set Enrichment Analysis for Gene Ontology (biological process) clusters for the 167 Btk-dependent transcripts found in larvae CNS: Differentially expressed transcripts (167) were subjected to GSEA and enriched clusters were found. The genes belonging to respective cluster are marked in either red (up-regulated) or blue (down-regulated) depending on the direction of the gene. For the main Figure 5, Figure S2 shows the respective genes found for each cluster (grey-zoned in Figure 5). The genes belonging to respective cluster are marked in either red (up-regulated) or blue (down-regulated) depending on the direction of the gene.
Figure S3 Gene Set Enrichment Analysis for Gene Ontology (biological process) clusters for the 744 Btk-dependent transcripts found in adult head tissue: Enriched clusters of Biological Processes were found in the Btk-dependent Adult head tissue analysis and were statistically significant. The genes belonging to respective cluster are marked in either red (up-regulated) or blue (down-regulated) depending on the direction of the gene. For the main Figure 6, Figure S3 shows the respective genes found for each cluster (grey-zoned in Figure 6). The genes belonging to respective cluster are marked depending on the direction of the gene. For the main Figure 6, Figure S3 shows significant. The genes belonging to respective cluster are marked dependent Adult head tissue analysis and were statistically significant. The genes belonging to respective cluster are marked dependent Adult head tissue analysis and were statistically significant.

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Table S1 Table S1 mirrors Table 1 (25 genes found to overlap between D. melanogaster larval CNS and adult head in addition of adding the dimension of every gene’s Gene Ontology term including Gene Ontology ID.

Author Contributions
Conceived and designed the experiments: JML, CIES. Performed the experiments: HNM, PK. Analyzed the data: JML, HNM, CIES. Contributed reagents/materials/analysis tools: ND Y4. Wrote the paper: JML, HNM, CIES.