cd1 Mutation in Drosophila Affects Phenoxazinone Synthase Catalytic Site and Impairs Long-Term Memory

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Abstract: Being involved in development of Huntington’s, Parkinson’s and Alzheimer’s diseases, kynurenine pathway (KP) of tryptophan metabolism plays a significant role in modulation of neuropathology. Accumulation of a prooxidant 3-hydroxykynurenine (3-HOK) leads to oxidative stress and neuronal cell apoptosis. Drosophila mutant cardinal (cd1) with 3-HOK excess shows age-dependent neurodegeneration and short-term memory impairments, thereby presenting a model for senile dementia. Although cd gene for phenoxazinone synthase (PHS) catalyzing 3-HOK dimerization has been presumed to harbor the cd1 mutation, its molecular nature remained obscure. Using next generation sequencing, we have shown that the cd gene in cd1 carries a long deletion leading to PHS active site destruction. Contrary to the wild type Canton-S (CS), cd1 males showed defective long-term memory (LTM) in conditioned courtship suppression paradigm (CCSP) at days 5–29 after eclosion. The number of dopaminergic neurons (DAN) regulating fly locomotor activity showed an age-dependent tendency to decrease in cd1 relative to CS. Thus, in accordance with the concept “from the gene to behavior” proclaimed by S. Benzer, we have shown that the aberrant PHS sequence in cd1 provokes drastic LTM impairments and DAN alterations.

Keywords: Drosophila; cardinal; 3-hydroxykynurenine; phenoxazinone synthase; courtship suppression; long-term memory; dopaminergic neurons

1. Introduction

About half a century ago, Prof. I.P. Lapin discovered the neurotropic effects of kynurenines and suggested the “serotonin-kynurenine hypothesis of depression” [1,2]. Activation of indoleamine 2,3-dioxygenase (IDO), the main enzyme of the kynurenine pathway (KP), decreases the brain serotonin level in favor of kynurenine (KYN) and its products, collectively called kynurenines. It enhances the level of stress and anxiety, being an adaptive reaction aimed at provoking a defensive behavior, but in excess may lead to heavy depression and even to suicide attempts [3]. IDO is induced by pro-inflammatory cytokines during brain infections and neurodegenerative disorders, such as Huntington’s, Parkinson’s and Alzheimer’s diseases [4,5]. Imbalance of KP products affects neuropathology. Accumulation of a prooxidant 3-hydroxykynurenine (3-HOK) leads to oxidative stress and thereby all the subsequent KP stages. This does not affect NAD+ cofactor synthesis [7]. Insects lack quinolinic acid production and neuronal cell apoptosis.

KP is the major path of tryptophan catabolism. In mammals, it leads to nicotinamide adenine nucleotide (NAD+) cofactor synthesis [7]. Insects lack quinolinic acid production and thereby all the subsequent KP stages. This does not affect NAD+ level in the body [8]. This is a great advantage for studies on the molecular mechanism of kynurenines’ biological activities while using insect models, such as the Drosophila melanogaster KP mutants [9]. For insects, KP is the source of the brown eye pigment xanthommatin (XAN) synthesized from...
3-HOK upon its oxidative autodimerization [8,10]. In *Drosophila*, mutations at different KP enzymatic stages affect the eye color (becoming bright red) along with accumulation of specific XAN precursors and lack of products downstream the mutation (Figure 1). *vermilion* (v) inactivates the key KP enzyme tryptophan 2,3-dioxygenase (TDO), resulting in 6-fold rise of tryptophan level [11]. In *cinnabar* (cn), dysfunction of kynurenine monooxygenase (KMO) leads to 2-fold rise in KYNA and 1.5-fold rise in KYN levels with subsequent lack of 3-HOK [12]. In *cardinal* (cd), dysfunction of phenoxazinone synthase (PHS) that governs 3-HOK enzymatic dimerization leads to 2.9-fold increase in 3-HOK level [13,14]. XAN can be produced non-enzymatically due to 3-HOK spontaneous autodimerization [15,16], resulting in the eye color darkening soon after eclosion; Xanthurenic acid (XAA) and cardinalic acid are also synthesized from 3-HOK. The levels of 3-hydroxyanthranilic acid (3-HAA) and anthranilic acid are low in *Drosophila* [8], making *cd* a useful model for specific studies of 3-HOK neurotropic effects.

![Figure 1. Kynurenine pathway in Drosophila.](image)

3-HOK and 3-HAA possess antioxidant activity via inhibiting lipid peroxidation, while the antioxidant power of XAA is significantly less [17,18]. However, in high concentrations, 3-HOK undergoes non-enzymatic oxidative autodimerization, leading to overproduction of reactive oxygen species (ROS), such as hydrogen peroxide inducing apoptosis in neuronal cell culture [19,20]. Total antioxidant activity decreases in *cd* heads compared to CS, while their lipid peroxidation also shows a tendency to decrease with age [16]. This reflects the
dual 3-HOK effects on redox processes [21]. KYNA is a non-specific inhibitor of ionotropic glutamate receptors (iGluRs) [22] that ameliorates excitotoxicity caused by glutamate excess. KYNA can form complexes with both mammalian and Drosophila iGluR [23], also serving as an α7 nicotinic acetylcholine receptor antagonist [24].

The high 3-HOK/KYNA level in htt mutant, a Drosophila model for Huntington’s disease, is one of the most crucial factors for neurodegeneration development [25,26]. Rise in 3-HOK/KYNA in flies also leads to age-dependent memory impairments. On days 12–29 of the adult life, cd1 shows the decrease in learning and 3 h short-term memory (STM) compared to CS in the conditioned courtship suppression paradigm (CCSP). On days 21–29, cd1 demonstrates the development of calyx synaptic pathology. cn1 does not show the age-dependent STM decay and synaptic pathology, being similar to CS [27]. In aged flies, the interpulse interval variance, a marker of neurodegeneration, is the highest for cd1 and lowest for cn1. In young flies, similar differences in heat shock-induced brain apoptosis are observed [28]. Thus, 3-HOK accumulation in flies has a general neurotoxic effect progressing with age.

The naive (without courtship experience) males of CS, cn1 and cd1 have high courtship indices (CI) up to day 29, indicating that their responses to pheromones and visual cues from a fertilized female stay unchanged [27]. At the same time, spontaneous locomotor activity significantly differs in the middle-aged flies. On days 13–29, running speed and run bout time decrease while run frequency increases for cn1 compared to CS; for cd1 the changes are the opposite. On day 40, the cd1 index of activity specifically decreases, possibly due to age-dependent neurotoxicity development [29]. Thus, it is crucial to differentiate KYNA and 3-HOK effects on spontaneous locomotion activity, courtship activity, memory processes and neurodegeneration.

Although cd1 mutation has been known about since the birth of Drosophila genetics [30] and is used as a marker gene in many strains, its molecular nature remains unknown. PHS activity associated with vesicular structures in the pigment cells called the pigment granules specifically rises at the pupal stage when XAN synthesis occurs. This activity decreases both in cd and a number of other XAN-deficient strains, such as v and cn mutants, possibly due to the lack of 3-HOK that may activate PHS [31]. According to other researchers, 3-HOK non-enzymatic dimerization decreases in head homogenates of the cd strain, while 3-HOK enzymatic dimerization does not change compared to the wild type [32]. However, they estimated the “PHS activity” in a soluble fraction that lacked the pigment granules. Hence, it was probably the non-enzymatic 3-HOK dimerization activity. The role of PHS in Drosophila 3-HOK dimerization was proved using the transgenic wild type cd gene, the expression of which completely restores the ommochrome synthesis in several loss-of-function cd mutants [33].

Drosophila PHS is predicted to have N-terminal transmembrane domain and heme peroxidase domain. Being localized in pigment cells, namely in type I pigment granules, PHS prevents Ago2 loading to protein-siRNA complex. siRNA-mediated silencing is required for normal vesicle trafficking and ommochrome synthesis. At the same time, mutations in the v, cn and cd genes specifically inhibit this silencing pathway in the fly eyes [33]. This indicates positive feedback between the ommochrome level and pigment granule trafficking. Surprisingly, the level of protein-bound 3-HOK (PB-3-HOK) in the cd1 brains and head capsules appears to be several times lower compared to CS, though it remains higher compared to v1 and cn1 [29]. Hence, Drosophila PHS may also participate in 3-HOK conjugation to some proteins.

To reveal the molecular nature of cd1 mutation, we performed the next-generation sequencing (NGS) of the cardinal gene (cd) for CS and cd1 strains. We also studied the relative abilities of CS, cn1 and cd1 to learn and form LTM in CCSP. The age-dependent changes in learning and memory abilities were estimated for flies trained on days 5, 13, 21 and 29 after eclosion. The number of tyrosine hydroxylase (TH)-positive dopaminergic neurons (DAN) within the brain areas regulating courtship memory and locomotor activity was counted in young and aged CS and cd1.
2. Results

2.1. PCR Mapping of the cd Gene

To reveal possible cd allelic variants in Drosophila populations, PCR mapping of the cd gene was performed for two different single males of CS and cd1 strains (Figure 2). All bands were present in CS, their size being approximately equal to the theoretically expected value (see Supplementary Materials, Text S1). Band 3 was weak in CS #2, which might reflect some primer non-specificity. Similar bands were observed in cd1. However, band 5 was nearly absent in cd1 #2. There was a smeared band below, which could also be seen in cd1 #1. Hence, the cd gene in cd1 seems to carry some polymorphism, probably a deletion, within area 5.

![Figure 2. PCR mapping of the cd gene in CS and cd1 mutant. 0: 1 kb ladder. 1–7: the primer pairs (f—forward, r—reverse). Positions on the X chromosome are shown in kb (FlyBase data). 8fr—the amplicon III (see the text).](image)

The presence of deletion was confirmed for the cd1 amplicons, including the whole cd gene with the flanking sequences (Figure S1). The amplicon I (1f–7r) in CS was compared to the amplicon I' in cd1 (8f–7r) with nearly matching borders and was found to be longer, and the band I' was double. Surprisingly, there were no visible interstrain differences for the amplicon II (2f–7r) lacking cd5' end, probably corresponding to a different copy of the cd gene. Thus, cd1 seems to carry at least two inactive copies of cd, one of which lacks a significant part of the gene.

2.2. The cd Gene Sequencing in CS and cd1

The results of sequencing of four amplicons are presented in Figure 3. For the wild type strain, only one amplicon (I) covering the whole gene with flanking sequences was assayed. Compared to the reference genome sequence, CS carried ~50 single nucleotide
polymorphisms (SNPs) within the cd gene and flanking 5’ and 3’ areas (Table S1). Most of them were synonymous, some were upstream gene variant modifiers, being localized within introns and the intergenic spacer, and four were missense mutations leading to amino acid substitutions. All of them were beyond the predicted PHS active site and presumably could not affect its function. There were also two (19 bp and 2 bp) A/T-rich indels downstream cd. Sequencing depth (DP) was similar for all positions. C(1504)/T SNP within the second part of the CS 3r primer binding site probably caused the non-optimal amplification of band 3 in this strain.

The picture was significantly different for cd1. Here, the amplicon I’ (8f–7r), the borders of which nearly matched those for amplicon I (1f–7r), was analyzed. There was a drastic drop of DP beginning from 1944 bp till the reverse primer binding site, being the evidence of the large deletion, though some areas showed local increase in DP starting from ~200 bp after the beginning of the deletion. The non-zero DP of the cd 3’ area corresponded to the double band I’ with the faint heavy part of the band. Thus, it was hard to localize the 3’ end of deletion for this amplicon. The sequence of amplicon II was nearly the same as for CS, except that the cd 5 end was not amplified in II, and SNP distribution in I (CS) and II (cd1) significantly differed from that of I’ (cd1). There was no evidence of a long deletion such as in I’: DP was similar for all positions. This confirms that II is a part of the second inactive cd copy in cd1.

To identify the 3’ end of the deletion within the cd gene in cd1, we performed additional assay for amplicon III (8f–8r) that was obtained independently for the other fly samples and was significantly longer than I and I’. Again, we observed a significant drop of DP from 1944 bp, up to 2080 bp (including the AGGTGG... TTCAAT area; see Text S2). The deletion was flanked by GG/CC repeats. This makes possible formation of a loop with a stem including A/T pair of the deleted sequence. The deletion was located within band 5 (1716–2279 bp). On PCR in cd1, the light smeared part of the double band 5 was several hundred bp shorter compared to the predicted size (see Figure 2). T(1726)/G SNP was within 5f primer binding site in cd1; hence the double band also might be an effect of non-specific amplification.

The found deletion covered 137 bp of the cd gene, including the key residues of the active site (Figure 4), such as Arg 333/542, Asn 420/629, Arg 423/632 (heme fixation) and His 336/545 (interaction with the heme group, participation in catalysis). The residue numbers are given according to their order in the PHS model structure/full protein sequence; see also [16] for the description of PHS active site and catalytic mechanism. Moreover, the deletion induced a frameshift and synthesis of the abnormal 10 AA peptide (AILALGGAWH) followed by translation termination (Figure S2). As a result, the mutant PHS should lack a C-terminal part including most residues of the active site. Both I’ and III amplicons also had T(20) > G substitution within the binding site of the transcription factor Adf-1, which might affect cd expression in cd1.

In summary, cd1 carries at least two non-functional cd copies—cd(A) with T(20) > G change in the promoter area and the deletion disrupting the enzyme catalytic site, and cd(B), for which the molecular nature of impairment remains unknown.
Figure 3. Aligned reads of the cd gene amplicons. Reads are shown in grey. Single nucleotide polymorphisms (SNPs) and misaligned reads are shown in other colors. DP—sequencing depth, del—deletion. Positions on the X chromosome are shown in kb. The amplicons are indicated by Roman numerals.
The found deletion covered 137 bp of the cd gene, including change in the promoter area and the deletion disrupting the enzyme catalytic site, and cd1 mutation impairs cd(A) synthesis and maturation, or cd(A) mRNA decrease is compensated by cd(B) transcription activity.

2.3. The cd Gene Expression in Late Pupae

To reveal whether cd1 mutation impairs cd mRNA synthesis, we performed a semi-quantitative RT-PCR analysis of cd expression in both strains at the late pupal stage, when PHS is actively involved in XAN production. We did not observe a decrease in cd activity in cd1 compared to CS, both for the total RNA and poly(A) form (Figure 5). Thus, either T(20) > G substitution in cd1 does not impair cd(A) synthesis and maturation, or cd(A) mRNA decrease is compensated by cd(B) transcription activity.

![Three-dimensional model structure of the Drosophila phenoxazinone synthase (PHS) active site.](image)

**Figure 4.** Three-dimensional model structure of the Drosophila phenoxazinone synthase (PHS) active site. (a) CS; (b) cd1 (amplicon III).

2.4. Learning and Long-Term Memory Formation

The learning acquisition and long-term memory (LTM) retention were assessed for Drosophila males in CCSP at 5, 13, 21 and 29 days of adult life. On day 5, both the wild type strain CS and the mutant strain cd1 showed courtship index (CI) decrease just after training (0D). The difference between CI of naive and trained flies gradually becomes insignificant with age and time after learning, indicating memory decay (Figure S3). In CS, the learning index (LI) calculated at 0D point (LI(0D)) remained significantly different from zero up to 21 days after eclosion (Figure 6). On day 21, LI(2D) and LI(8D) did not differ from zero, and LI(8D) was significantly lower than LI(0D). The above indicated 2D and 8D LTM were impaired in middle-aged CS. In cd1, both 2D and 8D LTM were absent during the life span, along with the intact learning ability. On day 5, cd1 showed LI(8D) decrease compared
to CS. On day 29, LI did not statistically differ from zero for both strains and time points, except for cd\(^1\) LI(0D), indicating preservation of its ability to learn while training for 5 h. In the other KP mutant cn\(^1\), both learning ability and 2D LTM were preserved up to 21 days, while 8D LTM was impaired during the whole studied life period (Figure S4). Thus, both KP mutants showed 8D LTM impairments, while cd\(^1\) also lacked 2D LTM starting from a young age.

Figure 6. Learning and long-term memory retention in CS and cd\(^1\). X axis: the age where training was performed (days), Y axis: learning indices (LI), %. 0D—immediately after training (learning); 2D—2 days after training; 8D—8 days after training. Statistical differences: # from CS; $ from 0D, * from zero (two-sided randomization test; \(p < 0.05\), \(n = 20\)).

2.5. Dopaminergic Neurons in Adult Drosophila Brain

A 24 h LTM formation in CCSP paradigm is known to depend on aSP13 DAN, innervating the tip of the mushroom body (MB) fru\(^+\)\(\gamma\)-lobe [34]. aSP13 neurons belong to the protocerebral anterior medial (PAM) cluster. Among the other DAN innervating MB are the protocerebral posterior lateral (PPL) and medial (PPM) clusters [35]. The scarlet mutant with a defect in 3-HOK transport to pigment cells is characterized by ROS increase, decreased climbing index and age-dependent loss of DAN belonging to a PPL1 cluster [36]. It is reasonable to assume that 3-HOK accumulation in cd\(^1\) leads to age-dependent PAM degeneration, possibly causing LTM impairment. To check this hypothesis, we counted the cell number in several DAN clusters for CS and cd\(^1\) on days 5 and 29.

The distribution of tyrosine hydroxylase (TH)-positive DAN clusters in the 5-day-old and 29-day-old Drosophila brains is shown in Figure 7. As expected, the cell bodies of the
TH-positive neurons were located above the antennal lobes (AL), near the superior medial protocerebrum (SMP). Their processes innervated the horizontal lobes of the MB, especially the medial tip of the $\beta'$ lobe ($\beta'$L), like in [35], where the processes extending from both hemispheres join together. The semicircular TH-positive tracts that surrounded SMP were less visible for GFP-positive cells, whereas the tracts connecting PAM cells to $\beta'$L were seen better (Figure S5). PPL1 and PPM2 neurons were located lateral and medial to calices (Cal), whereas PPM3 neurons were located somewhat below Cal. Cell bodies formed clusters tightly connected in groups by their processes. In each hemisphere, the PAM cluster was subdivided into two approximately equal branches (see 3D reconstruction in Figure S5).

Figure 7. The tyrosine hydroxylase (TH)-positive clusters in the adult CS and cd1 brains. (a). PAM clusters of young (5 day-old) and aged (29 day-old) CS and cd1 (anterior side, X 63, scale bar 50 µm). (b). PPL1, PPM2 and PPM3 clusters of 5-day-old CS (posterior side, X 63, scale bar 50 µm). (c). PPL1, PPM2 and PPM3 clusters of young and aged CS and cd1 (X 20, scale bar 200 µm). Color scheme: cyan—neuropil (CSP); red—TH (DAN); blue—nuclei. AL—antennal lobe; Cal—calyx; PB—protocerebral bridge; SMP—superior medial protocerebrum. * TH-positive clusters. See abbreviations in the text.
The average numbers of TH-positive cells on days 5 and 29 of the Drosophila adult life were somewhat lower compared to previous data [35], probably due to lower resolution in our case. Mao and Davis gave the approximate values of 100, 12, 8 and 6–8 for PAM, PPL1, PPM2 and PPM3 neurons, respectively, whereas in our study these clusters in the young CS included approximately 43, 10, 6 and 5 neurons (Figure 8). Other DAN clusters were not considered. Proper orientation of the brains was reported to be crucial for reproducible quantification of DAN [37]. For the Gal4 > UAS strain expressing GFP in DAN and serotoninergic neurons (SRNs), the number of cells in PAM clusters was larger: 74 on average and up to 100. SRNs might have only a minor impact on this number [38,39].

We did not observe age or strain-specific differences in the average number of PAM cells responsible for courtship memory, as well as in PPM3 cells innervating the central complex (CC). The average number of PPM2 cells was slightly increased in 5-day-old cd¹ relative to CS. On day 29, the number of PAM cells was non-significantly decreased in cd¹ relative to the wild type strain, while the decrease of PPL1 cell number was nearly significant (p = 0.057). The observed tendency probably reflected the age-dependent DAN degradation in cd¹ due to oxidative stress.

![Graph showing the average number of TH-positive neurons in the Drosophila DAN clusters.](image)

**Figure 8.** The average number of TH-positive neurons in the Drosophila DAN clusters. X axis: age of the fly (days); Y axis: cell number (N). Statistical difference: # from CS; two-sided randomization test, p is shown above the chart columns, n is marked with white numbers on the columns.

### 3. Discussion

The gene-to-behavior approach suggested by S. Benzer in 1971 raises the question how the one-dimensional information contained in genes is translated into a multi-dimensional spatial structure of the central nervous system realized in complex behavioral patterns [40]. The Drosophila KP mutants serve as an excellent model to investigate the causal chain connecting gene structure changes to physiological and behavioral alterations. Among them is the cd¹ mutation. cd¹ has not been sequenced up to date, despite being actively used as a marker gene and in HD modeling.

Our NGS data on the cd¹ structure revealed the complex nature of this mutation. There are at least two different cd copies in the genome of the cd¹ flies, both inactive or only partially active. One mutant copy, cd¹(A), carries a deletion disrupting PHS active site and an SNP within the predicted Adf-1 binding site. Another copy, cd¹(B), is similar to the wild type cd, except for its 5' end that has not been sequenced. As amplicon I contains a 5' end and lacks the middle part of amplicon II, we suppose that cd¹(B) may carry some alterations in the 5' area. The double bands for 1f–7r and 8f–7r amplicons in cd¹ may also indicate some polymorphisms within the 1f/8f binding area. We did not observe different eye color phenotypes in the cd¹ population; hence, both cd¹(A) and cd¹(B) are recessive and do not recombine to produce the wild type cd form. As cd¹ was cantonized before experiments and SNP distribution is very similar in cd¹(B) and CS, cd¹(B) may be the non-functional part of the wild type cd gene obtained in the process of cantonization.
spatial structure of the central nervous system realized in complex behavioral patterns [40]. The *Drosophila* KP mutants serve as an excellent model to investigate the causal chain connecting gene structure changes to physiological and behavioral alterations. Among them is the cd mutation. cd1 has not been sequenced up to date, despite being actively used as a marker gene and in HD modeling.

Our NGS data on the cd1 cd structure revealed the complex nature of this mutation. There are at least two different cd copies in the genome of the cd1 flies, both inactive or only partially active. One mutant copy, cd(A), carries a deletion disrupting PHS active site and an SNP within the predicted Adf-1 binding site. Another copy, cd(B), is similar to the wild type cd, except for its 5' end that has not been sequenced. As amplicon I contains a 5' end and lacks the middle part of amplicon II, we suppose that cd(B) may carry some alterations in the 5' area. The double bands for 1f–7r and 8f–7r amplicons in cd1 may also indicate some polymorphisms within the 1f/8f binding area. We did not observe different eye color phenotypes in the cd1 population; hence, both cd(A) and cd(B) are recessive and do not recombine to produce the wild type cd form. As cd1 was cantonized before experiments and SNP distribution is very similar in cd(B) and CS, cd(B) may be the non-functional part of the wild type cd gene obtained in the process of cantonization.

There can be two different ways in which both cd copies are arranged in the genome of cd1 (Figure 9). In the first case (Figure 9a), they are located in one locus and can be redistributed in the population in different combinations. In the second case (Figure 9b), two different cd copies are located in different parts of the genome, within the 3R:94A1-94E2 cytogenetic region (Flybase data). For single males, we observed differences in the brightness of band 5, which might indicate the presence or absence of short (A) and long (B) cd forms (see Figure 2). Both II and III cd amplicons are followed by the Cyp6d4 gene, indicating their equal position in the genome. These facts argue in favor of the first case (Figure 9a). Alternatively, there may be a long insertion in the cd1 genome carrying both cd and Cyp6d4, where cd is represented by A or B forms. In our further research, we plan to perform in situ hybridization to visualize the deleted fragment of the cd gene on the cd1 polytene chromosomes to check whether it resides only in half of chromatids at cd locus (case 1), or in all chromatids but at some different loci (case 2). Isolation of different stocks and sequencing is also possible in the future.

![Figure 9](image-url)  
**Figure 9.** Possible arrangement of different cd copies in the genome of cd1 flies. Grey rectangle is either cd (case a) or cd followed by Cyp6d4 (case b). A and B—two variants of cd; del—deletion; ?—the presumable alterations within cd(B) 5’ area.
The deletion of the 3’ end covers a significant part of PHS active site, probably leading to a drastic decrease in PHS catalytic activity and 3-HOK accumulation. Both facts are in agreement with experimental data [13,14,31]. Here, we have shown that the level of cd RNA at the late pupal stage was not decreased in cd compared to CS. PHS may also preserve some functional activity, causing the cd eye darkening soon after eclosion—the effect usually explained by 3-HOK autodimerization.

The prolonged action of 3-HOK induces the oxidative stress and neuronal apoptosis [19,20]. Seemingly, this causes functional disturbances in cd manifestations, such as courtship song defects [28], and possibly STM disturbance in aged flies [27]. Heat shock induces apoptosis in young cd flies [28]. In addition, it impairs STM in cd [41]. In contrast, cn1 shows the neuroprotective and anti-apoptotic activity [28]. In our study, cn1 demonstrated normal 2D STM in CCSP up to day 21 of adult life, but both KP mutants had impaired 8D LTM. The lack of 8D LTM was also shown for 5-day-old v1 with absence of kynurenines and intact 3-h STM [42].

The mechanisms of CCSP are complex and are still not fully understood [43]. Figure 10 gives a simplified scheme of the brain areas participating in courtship behavior [34,44,45]. Let us highlight several key elements crucial for CCSP memory formation:

1. The olfactory sensory neurons Or67d for anti-aphrodisiac cis-vaccenyl acetate (cVA), which is crucial for courtship suppression [34].
2. The visual projection neurons (VPN) from the optic lobe (OL).
3. MB calyx (Cal) and intrinsic neurons. MB are responsible for the classical olfactory conditioning [46] and are not required for leaning in CCSP [47,48]. However, Kenyon cells (KCs) of MB receive inputs not only from the olfactory AL. Subesophageal ganglion (SEG) responsible for taste sensitivity activates the dorsal accessory calyx (dAC) [49]. yd neurons of the ventral accessory calyx (vAC) participate in CCSP STM

Figure 10. The simplified scheme of the Drosophila brain structures regulating courtship and memory formation in conditioned courtship suppression paradigm (CCSP). Abbreviations are given in text. Nerve cell bodies are shown as circles. Specific neurotransmitters are shown by colors.

1. The olfactory sensory neurons Or67d for anti-aphrodisiac cis-vaccenyl acetate (cVA), which is crucial for courtship suppression [34].
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formation, receiving visual inputs from OL [48,50]. These neurons project to the MB γ5 area, crucial for courtship suppression [51].

4. The intrinsic neurons of MB projections to the MB output neurons (MBONs). Their synaptic contacts are tightly regulated by DANs, activated by unconditioned stimuli, such as electric shock or sugar. They do not encode specific modality of a stimulus, rather its positive or negative valence. After learning, the mutual MBON effect is shifted towards attraction or avoidance, biasing the fly’s behavior [52].

5. MBON project to the higher brain integrative centers, such as SMP, lateral protocerebrum (LP) and lateral horn (LH), also containing about 90% of the DANs dendritic arbors [50,52]. LP is the major site for the integration of discrete sensory stimulus, aggregated by P1 cluster, which triggers male courtship [44,45].

In our study, we used a CCSP retraining protocol, where a mated female served a trainer [53,54]. According to Keleman and coauthors, courtship learning in this case occurs as an enhanced response of a male after unsuccessful courtship to cVA released from a female. The active rejection by a female is necessary for training, while cVA is required for memory performance. aSP13 DANs of a PAM cluster play a key role in CCSP. They innervate the tip of the MB γ-lobe (γ5 area), expressing dopamine receptors DopR1 [34]. The other name of aSP13 is PAM-γ5 [51]. aSP13 prolong potentiation of MBγ-M6 MBON synapses, being the physical basis of the courtship STM [55]. The prolonged aSP13 activation leads to protein-synthesis-dependent LTM consolidation, dependent on the Orb2 protein [56,57].

Additional MB structures may participate in memory retrieval. Blocking the output of γ neurons disrupts STM, leaving LTM intact, while some other MB neurons, such as the neurons of αβ lobes, probably govern LTM expression [54]. Sleep is the other factor crucial for memory consolidation in CCSP. aSP13 are activated after courtship by a specific class of sleep-promoting vFB neurons in the fan-shaped body of the central complex (CC). An activation of vFB neurons at a specific time interval after learning is sufficient for LTM consolidation [58].

Our data also suggest different mechanisms for the Drosophila STM and LTM. In young flies, 8D LTM was absent in both KP mutants and v1, whereas 2D LTM was intact in v1 and cn1, and 3-h STM was intact in all the studied strains [27,42]. LTM preservation up to day 8 seems to require robust memory mechanisms impaired to varying degrees in KP mutants. Although KYNA accumulation in cn1 has neuroprotective effects, it also damages LTM storage or retrieval 8 days after learning, probably due to KYNA inhibitory action on iGluR or nicotinic receptors. M6-MBONs are putatively glutamatergic neurons [51], so KYNA excess can inhibit the output from the γ5 area and/or its translocation to other MB regions, crucial for LTM expression. cd1 with accumulation of the neurotoxic 3-HOK shows the most pronounced memory defects. The lack of 8D memory in cd1 may reflect the faster decay of its LTM compared to v1 and cn1. The interstrain differences were seen on days 5 and 13. At the same time, STM decrease and calyx neuropathology was observed only in aged cd1 [27]. Hence, LTM impairments in cd1 are ahead of the visible brain damage development and possibly occur at a more subtle neuronal level. As STM is also impaired in 5-day-old cd1 after heat shock, 3HOK-dependent neurotoxicity seems to occur early enough, being manifested under special conditions.

DAN clusters that regulate CCSP memory formation and consolidation may be selectively susceptible to the toxic effects of 3-HOK. However, we did not see any difference in PAM number for young Cs and cd1. A slight increase in PPM2 number was observed in aged cd1. These neurons innervate the part of the medial protocerebrum and SEG [35]. In aged flies, there was a trend of PAM decrease in cd1 relative to Cs. However, the difference was non-significant, and LTM was equally impaired in both strains. As the number of PAM neurons did not change in Cs upon aging, the LTM impairments on day 29 seem not to be due to death of PAM neurons. However, we cannot rule out that LTM lack may be caused by the decrease of PAM activity. The total number of TH-positive DAN did not change in several Drosophila models for PD, though their functional activity decreased in
aged flies [37]. At the same time, both the loss of PPL1 neurons and locomotor defects were shown for scarlet mutant, a PD model with 3-HOK accumulation and increased ROS level [36]. In 29-day-old cd, we also observed a trend of PPL1 number decrease relative to CS. This trend may become even stronger in 40-day-old cd with a decrease in spontaneous locomotor activity [29]. While PAM is needed for courtship memory, PPL1 is unlikely to be involved in it [48].

OL is connected to the γ5 area responsible for courtship memory [48,50]. Hence, visual signals from a female may be important for the courtship suppressions. Environmental light is also critical for LTM, which is impaired when flies are constantly kept in darkness after training. Memory consolidation is regulated by the light-driven activity of the transcription factor CrebB in αβ and γ lobes of MB. CrebB activity in αβ lobes is also required for LTM maintenance [59].

All the studied KP mutants have impaired synthesis of brown eye pigment XAN and its derivatives. In cd, XAN is synthesized soon after eclosion, either due to 3-HOK non-enzymatic autodimerization, or due to PHS residual activity. All KP mutants showed a decrease in the PB-3-HOK form, both in their brains and head capsules [29]. The physiological role of such a 3-HOK form is not clear, but it may have some protective or regulatory functions. Since courtship activity is the same for KP mutants and does not change with age, the visual system regulating male orientation toward females seems to be unaffected [27]. However, some minor disturbances of visual processing due to pigmentation defects or 3-HOK-dependent neurotoxicity may be crucial for LTM formation and preservation.

In summary, the deletion of the part of the cd gene in cd encoding the PHS catalytic site explains the three-fold increase in free 3-HOK that was previously shown for this mutant. Some residual PHS activity may preserve being possibly associated with the other cd copy. Checking this assumption needs further research, as well as mapping different cd copies in the cd genome. It also remains to be found whether PHS plays some role in 3-HOK conjugation to proteins. Studying the role of DAN in 3-HOK-induced memory disturbances requires specific suppression of DAN cluster activity in cd. The other task for the future is checking the influence of visual capacity on the abilities of KP mutants to form and preserve LTM. Disclosing mechanisms of behavioral impairments for the Drosophila KP mutants serving as model objects for different neural disturbances helps to uncover the molecular mechanisms of neurodegeneration and cognitive disorders.

4. Materials and Methods

4.1. Drosophila Strains

Fly strains were taken from Biocollection of the Pavlov Institute of Physiology Russian Academy of Sciences, Saint Petersburg, Russia. The following strains were used:

1. Canton-S (CS), the wild type strain; dark red eyes.
2. cinnabar (cn)—this carries a mutation in the kynurenine monooxygenase gene; lack of 3-HOK, KYNA excess, bright red eyes.
3. cardinal (cd); Bloomington Drosophila Stock Center, #3052)—this carries a mutation in the cd gene; increase in 3-HOK level, bright red eyes after eclosion; in 3-day-old flies the eye color becomes dark red.

cn and cd strains were out-crossed to CS for nine generations. All strains were raised on a standard yeast–raisin medium with 8 a.m.–8 p.m. daily illumination at 25 ± 0.5 °C.

4. Strains from Bloomington Drosophila Stock Center:

#7009: w [1118]; P[w+[mC] = Ddc-GAL4.L]Lmpt[4.36]. GAL4 is expressed in dopaminergic (DAN) and serotonergic (SRN) neurons.

#32186: w+[t7.7] w+[mC] = 10XUAS-IVS-mCD8::GFP]attP40. This contains GFP, the expression of which is driven by GAL4.
4.2. DNA Extraction

DNA was extracted from 10 females (for sequencing; 300 µL buffer) or single males (for PCR mapping; 200 µL buffer). Flies were homogenized in DNA extraction buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M EDTA (pH 8.0), 0.2 M sucrose, 0.5% SDS, 0.5% diethyl pyrocarbonate) and heated for 30 min at 65 °C. After addition of sodium acetate (pH 5.2, the final concentration 1.8 M), homogenates were incubated at 0 °C for 30 min and centrifuged at 14,000 × g (MPW-65R microcentrifuge, Warsaw, Poland). An equal volume of 96% ethanol was added to supernatant. DNA was precipitated by centrifugation at 14,000 × g for 5 min and washed with 70% ethanol. DNA concentration was measured at 260 nm using spectrophotometry (Eppendorf BioPhotometer, Hamburg, Germany).

4.3. Polymerase Chain Reaction

According to Flybase (www.flybase.org, accessed on 1 December 2021), cd location is 3R:22,694,959 . . . 22,697,990 [+]. Upstream cd, Nup133 gene is located (3R:22,690,607..22,694,772 [+]) encoding a Nup133-like nuclear pore complex protein. Downstream cd, Cyp6d4 gene is located (3R:22,698,319..22,700,307 [+]) encoding a heme binding protein that belongs to the cytochrome P450 family. To cover the cd gene with flanking 5′ and 3′ areas, a series of gene-specific primers was designed using NCBI Primer Blast tool and D. melanogaster genomic reference sequence. For all primers, the predicted melting temperatures are within 59–61 °C. DNA was amplified with Long PCR Enzyme Mix (ThermoFisher Scientific, Waltham, MA USA, #K0182) according to the manufacturer’s guidelines using Verity 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA USA). Primer sequences, positions of overlapping amplicons and PCR parameters are given in Supplementary Materials, Text S1.

4.4. DNA Purification and Sequencing

The PCR products were separated by agarose gel electrophoresis using Agagel Mini system (Biometra, Göttingen, Germany). The products of interest were extracted from gel using QIAquick Gel extraction kit 250 (QIAGEN, Cat. No 28706). DNA concentration before sequencing was 7–20 µg/mL. DNA amplicons were purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) and fragmented using dsDNA Fragmentase (NEB, Ipswich, MA USA). The fragmentation reaction was carried out in 10 µL volume for 10 min at 37 °C using 25 ng of purified DNA. The fragment-DNA libraries were prepared using TruSeq Nano DNA LT Library Prep Kit (Illumina, San Diego, CA USA), following the manufacturer’s protocol. The prepared libraries were sequenced on Illumina HiSeq 2500 system the paired-end 2x130 mode. The raw sequence data were deposited in NCBI, Sequence Read Archive, ID: PRJNA840853.

4.5. Bioinformatics Analysis

Assembly of sequences and annotation were performed using D. melanogaster genome reference (dm6 genome assembly; NCBI GenBank). FastQC [60] was used to check the quality of reads. Low-quality reads were deleted with the Trimmomatic tool [61] using the following filtration parameters: leading: 15; trailing: 15; sliding window: 4:22; minlen: 36 or 40. Sequence assembly was performed for paired reads with the help of the BWA-MEM algorithm [62] and samtools/bcftools utilities [63]. IGV software [64] was used for visualization of the sequencing data. Only the area corresponding to the amplicon position was analyzed, neglecting 5′ and 3′ regions with low sequence depth (DP). Substitutions with DP < 15 or quality < 33 (Phred33 quality score) within the amplicon area were omitted from the analysis. The effects of single nucleotide polymorphisms (SNPs) and indels were predicted with the help of SnpEff [65].

Search of transcription factor binding sites was performed with the help of PROMO 3.0 software [66]. Homology modeling of mutant PHS 3D structure was performed with MODELLER 9.14 software [67] using goat lactoperoxidase as a template (PDB ID: 2ojv). The modeling procedure of the wild type Drosophila PHS is described in detail in [16].
4.6. RNA Extraction, Reverse Transcription and Semi-Quantitative Real-Time Polymerase Chain Reaction (sq-RT-PCR)

RNA was extracted from late pupae (before eclosion). Four male pupae were homogenized in 300 µL of TRI reagent (MRC, TR 118). Total RNA was treated with DNase I in columns and purified using Direct-zol RNA MiniPrep (Zymo Research, R2050), in accordance with the manufacturer’s instructions. A total of 1 µg of RNA was used for cDNA synthesis with the help of MMLV reverse transcriptase (Evrogen, SK021). To assess the level of total RNA and the polyadenylated RNA form, we used random hexamer and oligo(dT) primers, respectively. Three biological replicates were made in each case. sq-RT-PCR was performed with the help of qPCR premix (Evrogen, PK156L), using StepOnePlus Real-Time PCR system (Applied Biosystems). cDNA for the \textit{Drosophila} ribosomal protein rpl32 served as an endogenous control. The \textit{cd} PCR amplicon spans the junction between exons 2 and 3. Primer sequences and PCR parameters are given in Text S3.

4.7. Long-Term Memory in Conditioned Courtship Suppression Paradigm

Learning acquisition and LTM retention was estimated in CCSP [53,68] modified for LTM [54]. For each strain, all males were divided into several independent groups: 1. Naive (without courtship experience); 2. 0D (learning, immediately after training with females); 3. 2D LTM (2 days after training); 4. 8D LTM (8 days after training). Each group consisted of four independent subgroups for training experiments at the age of 5, 13, 21 and 29 days after eclosion. Each subgroup contained about 20 flies. Males were kept separately in vials with standard food medium until the day of experiment. Fertilized 5-day-old CS females served as objects of courtship.

For training, a naïve male and a female were placed together into a food-containing vial for 5 h. The free space of movement was about 20 mm (diameter) \(\times\) 10 mm (height). After training, a male was either tested immediately with a new female (learning) or kept separately in a food-containing vial for 2 days (2D LTM) or 8 days (8D LTM). During test, a male and a female were placed together into a Perspex chamber (diameter 15 mm, height 5 mm). After 45 s of adaptation, the ethogram of a male courtship behavior was recorded for 300 s, manually fixing specific courtship elements (orientation, vibration, attempted copulation), as well as of elements unrelated to courtship (running, preening, rest). A specifically designed \textit{Drosophila} courtship Lite (DCL) software was used to decipher and analyze the recorded data (Kamyshev, 2006). The program is freely available from the author (nkamster@gmail.com) upon request.

To quantitatively estimate the learning acquisition and memory formation, courtship index (CI) was calculated for each male as a percentage of test time spent in courtship. Learning index (LI) was calculated using the formula:

\[
LI = \left[\left(\frac{\text{CI}_N - \text{CI}_T}{\text{CI}_T}\right) \times 100\%\right] = (1 - \frac{\text{CI}_N}{\text{CI}_T}) \times 100\%
\]

where \(\text{CI}_N\) is the average CI for naïve males, and \(\text{CI}_T\) is the average CI for males after training. The naïve and trained males were the same age.

Statistical analysis was performed using two-sided randomization test at significance level \(\alpha = 0.05\) [69] using DCL software. The following criteria of memory preservation were used: 1. LI was not significantly decreased with time compared to LI just after training. 2. LI was statistically different from zero. 3. LI for a mutant strain was not significantly decreased compared to the corresponding LI for the wild type strain CS.

4.8. Dopaminergic Neuron Visualization

The brains of 5-day-old and 29-day-old adult \textit{Drosophila} males were isolated in PBS buffer (pH 7.5) using fine needle-sharp style #5 tweezers (Merck, T4412) and fixed in 4% PFA on PBS for 1 h at RT, according to [70], without the freezing step before staining. The following antibodies were used: primary: rabbit anti-\textit{Drosophila} tyrosine hydroxylase (TH) antibody (Abcam, ab128249; dilution 1:200), mouse anti-\textit{Drosophila} cysteine string protein (CSP) antibody (courtesy of E. Buchner, Germany; dilution 1:20); secondary: goat
anti-rabbit Alexa Fluor 633 (Invitrogen, A21071; 1:200), goat anti-mouse Alexa Fluor 488 (Invitrogen, A32723; 1:200). Incubation was performed at 4 °C on a shaker for 3 days with primary antibodies and for 2 days with secondary antibodies. The long incubation period was used to improve the brain staining, similar to that in [29]. Cell nuclei were stained with DAPI (1.2 µg/mL PBS). The brains were mounted using Vectashield mounting medium (Vector laboratories) and scanned frontally by the confocal laser scanning microscope (LSM 710 Carl Zeiss; Confocal microscopy Resource Center; Pavlov Institute of Physiology Russian Academy of Sciences, Saint Petersburg, Russia). Scanning was performed using X20 objective (6 µm Z step) and X63 objective (2 µm Z step). Confocal images were analyzed using Fiji software. The number of TH-positive neurons was counted using Cell Counter plugin. TH-positive cells belonging to different DAN clusters were visually identified based on [35]. The average number of cells for each TH-positive cluster was counted for each brain hemisphere (or the number for one hemisphere, if the other was damaged). Statistical analysis was performed using two-sided t-test ($p < 0.05$).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232012356/s1.

Author Contributions: Conceptualization, A.V.Z. and E.A.N.; methodology, A.V.Z. and E.A.N.; formal analysis, A.V.Z., G.A.Z., P.N.I. and K.A.M.; investigation, A.V.Z., P.N.I. and K.A.M.; writing—original draft preparation, A.V.Z.; writing—review and editing, A.V.Z., E.A.N. and E.V.S.-P.; visualization, A.V.Z.; supervision, E.A.N., E.V.S.-P.; project administration, E.V.S.-P.; funding acquisition, A.V.Z. and E.V.S.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Russian Foundation for Basic Research grants No. 18-34-00761 (cd sequencing) and No. 20-015-00300 A (DAN staining, LTM analysis).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments: We thank D.E. Polev (“Serbalab”, Saint Petersburg, Russia) for the help in DNA sequencing. We also thank E. Buchner (Institute of Clinical Neurobiology, Würzburg, Germany) for the kindly provided Drosophila anti-CSP antibodies.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

AL—antennal lobe, Cal—calyx, CC—central complex, CI—courtship index, CCSP—conditioned courtship suppression paradigm, cd$^1$—cardinal$^1$, cn$^1$—cinnabar$^1$, CNS—central nervous system, CREB—cAMP response element-binding protein, CS—Canton-S, dAC—dorsal accessory calyx, CSP—cysteine string protein, DAN—dopaminergic neuron, DP—sequencing depth; 3-HAA—3-hydroxyanthranilic acid, 3-HOK—3-hydroxykynurenine, IDO—indoleamine 2,3-dioxxygenase, iGluR—ionotropic glutamate receptors, KC—Kenyon cell, KMO—kynurenine monooxygenase, KP—kynurenine pathway, KYN—kynurenine, KYNA—kynurenic acid, LI—learning index, LH—lateral horn, LP—lateral protocerebrum, LTM—long-term memory, MB—mushroom body, MBON—mushroom body output neurons, NAD$^+$—nicotinamide adenine nucleotide, OL—optic lobe, NGS—next generation sequencing, PAM—protocerebral anterior medial cluster, PB-3-HOK—protein-bound 3-hydroxykynurenine, PED—pedunculus, PHS—phenoxyazine synthase, PN—projection neurons, PPL—protocerebral posterior lateral cluster, PPM—protocerebral posterior medial cluster, ROS—reactive oxygen species, SEG—suboesophageal ganglion, SMP—superior medial protocerebrum, SNP—single nucleotide polymorphism, SRN—serotonergic neuron, STM—short-term memory, TDO—tryptophan-2 3-dioxxygenase, vAC—ventral accessory calyx, VPN—visual projection neurons, XAA—xanthurenic
acid, XAN—xanthommatin.

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