Disruption of aminergic signalling reveals novel compounds with distinct inhibitory effects on mosquito reproduction, locomotor function and survival

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Insecticide resistance amongst disease vectors is a growing problem and novel compounds are needed. Biogenic amines are important for neurotransmission and we have recently shown a potential role for these in mosquito fertility. Here, we dissected the relative contribution of different aminergic signalling pathways to biological processes essential for vectorial capacity such as fertility, locomotion and survival by injecting agonists and antagonists and showed that octopaminergic/tyraminergic signalling is essential for oviposition and hatching rate. We show that egg melanisation is regulated by adrenergic signalling, whose disruption causes premature melanisation specifically through the action of tyramine. In addition to this, co-injection of tyramine with DOPA, the precursor of melanin, had a strong cumulative negative effect on mosquito locomotion and survival. Dopaminergic and serotonergic antagonists such as amitriptyline and citalopram recapitulate this effect. Together these results reveal potential new target sites for the development of future mosquito sterilants and insecticides.
Results

Evolutionary relationships between selected human aminergic receptors and insect biogenic amine receptors. On the assumption that the level of evolutionary relatedness of different GPCRs should positively correlate with conservation of function and ligand partner, we performed a phylogenetic analysis of the different human, fruit fly and malaria mosquito GPCRs (Figure 2). As expected the aminergic GPCRs of two dipteran GPCRs were closer related to each other than to the human GPCRs and formed clusters that have been classified previously26–30. Our bootstrapping analysis further supported clades formed by the insect dopaminergic receptors (Dop1, Dop3), serotonergic receptors (5HT1,5,7) and α2 adrenergic-like octopamine receptors (OA3) with the respective human D1, D2, 5-HT and α2 adrenergic receptors. This was in concordance with pharmacological studies investigating GPCRs in insects which highlight potential functional similarities between human and insect GPCRs (Table 1). This close relationship between human and insect receptors could potentially limit the insecticidal application of molecules that target these GPCRs. Interestingly, none of the tyraminergic receptors (Tyr1-3) clustered together with the respective human adrenergic receptors, therefore molecules targeting those receptors might be more suitable to generate insect specific insecticides. Nonetheless, there are examples of clear differences in pharmacological responses of drugs against phylogenetically related receptors and even between different insect genera37. For example tick and Aedes mosquito D1 like receptors can be inhibited by Sch23390 while no effect is observed on the fruitfly38 or honey bee Dop1 receptor39. This highlights on one hand the opportunity to potentially generate mosquito specific agents but also the need to test empirically each compound on its merit for the species of interest.

Perturbation of the adrenergic system and dopamine availability inhibits female fertility. Our recent results showed that phenylalanine hydroxylase activity, which catalyzes the first step of phenylalanine metabolism by converting phenylalanine into tyrosine, is needed for oviposition and egg formation in An. gambiae mosquitoes8. Tyrosine is an essential precursor in the formation of insect neurotransmitters tyramine, octopamine and dopamine. In order to dissect the potential involvement of these biogenic amines in female mosquito fertility, we injected females with different agonists and antagonists of the adrenergic system and compounds involved in dopamine synthesis. The strongest effect on egg laying activity was observed by injection of tyramine, which resulted in complete inhibition of oviposition (Figure 3A). Oviposition rate was also reduced by 16% by the tyramine-derived neurotransmitter octopamine and by 28% by the α2-adrenergic agonist clonidine, compared to the PBS-injected control. We observed that relatively few compounds (octopamine, dobutamine and dopamine) had an effect on the number of eggs laid, possibly due to the fact that injection of compounds coincided with the latter stages of oogenesis, when oocytes would be expected to be fully formed (Figure 3B). However, many compounds (the α2-adrenergic agonist clonidine, α1-agonist prazosin, the β2-agonist clenbuterol and the DDC inhibitor carbipido) that had no effect on clutch size had a negative impact on embryo viability as measured by larval hatching rate (Figure 3C). Interestingly this effect seemed to be due to either the specific activation or inactivation of each of the different adrenergic receptors and the injection of compounds with putative opposing function on α2-receptors (yohimbine, antagonist), α1 receptors (phenylephrine, agonist) and β-receptors (acebutolol, sotalol, both antagonists) had no effect. Reduction in embryo viability by carbipido on the other hand could have been due to the fact that it targets dopa decarboxylase (DDC) which has a likely role in the formation of the embryo serosa, a protective membrane that can enclose the entire embryo39.

Tyramine injection induced a precocious oviposition egg melanisation phenotype which can be modulated via α-adrenergic inhibition. Since all tyramine-injected females failed to lay eggs, we dissected their ovaries to examine egg development. Most eggs retained by females appeared to be melanised (Figure 4A). This tyramine-
mediated premature melanisation phenotype was very unusual, as *An. gambiae* eggs ordinarily melanise only after oviposition. It is possible that the reduced oviposition phenotype in tyramine-injected females was directly due to premature melanisation of eggs and concomitant chorion hardening that prevented their physical release from the ovary rather than a behavioural effect on oviposition stimulus. Interestingly, when we injected DOPA or dopamine, known precursors of melanin synthesis, or any other adrenergic compounds, we did not observe this premature melanisation phenotype, suggesting a tyraminergic regulatory mechanism for this process (Figure 4B). To investigate this further we tried to rescue this melanisation effect in two ways: 1) by inhibition of melanin synthesis through injection of carbidopa that inhibits the dopa decarboxylase (DDC) essential for dopamine melanin and sclerotin synthesis; 2) antagonising injected tyramine activity with its antagonists prazosin or yohimbine. Fewer eggs were fully melanised in the presence of prazosin or yohimbine, suggesting a tyraminergic pathway is responsible for this effect (Figure 4C). We observed only a very low proportion of females was able to lay eggs following injection with any of the above compound combinations (Figure 4D) but we cannot exclude the possibility that general toxicity of these combinations contributed to low egg number and oviposition rate (Figure 4E). Carbidopa did not reduce tyramine-induced pre-oviposited egg melanisation but it did inhibit

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**Figure 2 | Phylogenetic analysis of selected human, fly and mosquito biogenic amine receptors.** Protein sequences of each receptor ("R") were first aligned in Muscle and then a Maximum-Likelihood tree was constructed in MEGA 6 using 1000-fold bootstrap re-sampling. All insect receptors are shown in green, while the human receptors are highlighted in blue. The numbers at the nodes of the branches represent the level of bootstrap support for each branch. The *D. melanogaster* FMRF amide receptor (DmFR, AAF47700.1) was used as outgroup. The accession numbers for each receptor are listed in Supplementary Table S1.
| compound       | function in humans                                                                 | function in insects                                                                 | Reference       |
|----------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-----------------|
| Tyrosine       | Precursor of DOPA, dopamine, adrenaline and noradrenaline                         | Precursor of DOPA, dopamine, tyramine and octopamine                              | [48]            |
| L-DOPA         | D(1-4) dopamine receptor agonist, dopamine precursor                               | Dopamine and DOPA melanin precursor,                                              | [48–50]         |
| Dopamine       | D(1-4) dopamine receptor agonist, dopamine transport inducer,                      | dopamine melanin precursor, Dap 1-3 receptor agonist,                             | [27,29,30,38,51,52] |
|                | dopamine beta hydroxylase ligand                                                   | Oct1-3 receptor agonist, Oct2R (OA3) agonist, TyR1 and TyR3 agonist               |                 |
| Carbidopa      | Aromatic amino acid decarboxylase (AADC) inhibitor                                | DDC inhibitor                                                                      | [53,54]         |
| Tyramine       | Trace amine associated receptor (TAAR) agonist, β-adrenergic receptor 1 and 2      | Oct1-3 receptor agonist, TyR1-3 agonist, Dop3 (D2 like) agonist                    | [29,30,38,45,55–57] |
| Octopamine     | TAAR agonist, ADRB2-antagonist, ADRB1,3-agonant                                    | Oct1-3 receptor agonist, TyR1 agonist, Oct1-3 agonist, Dop3 (D2 like) agonist      | [29,30,38,45,55–57] |
| Clonidine      | α2 adrenergic agonist                                                             | TyR agonist, Oct R agonist                                                        | [67,68]         |
| Yohimbine      | α2, 5-HT(1B), 5-HT(1D), and D(2) receptor antagonist, 5HT (1A) agonist            | ND                                                                                 | [69]            |
| Prazosin       | α1 antagonist                                                                     | ND                                                                                 | [70]            |
| Phenytoine     | α1 agonist                                                                        | ND                                                                                 | [71]            |
| Clenbuterol    | β2 agonist                                                                        | ND                                                                                 | [72]            |
| Dobutamine     | β1 and α1 agonist                                                                 | ND                                                                                 |                 |
| Acebutolol     | β1 antagonist                                                                      | ND                                                                                 |                 |
| Sotalol        | Non-selective β-blocker                                                           | Dap2 antagonist                                                                    | [27,73–77]      |
| Amtriptyline   | Noradrenaline and serotonin transport (SERT) inhibitor, 5HT-2A receptor antagonist, TrkA and TrkB receptor agonist | ND                                                                                 |                 |
| Sch-39166 (Ecopipam) | D1 receptor antagonist                                                                 | Dopamine 1 receptor (Dop1) agonist                                               | [78]            |
| SKF-38393      | D1 receptor agonist                                                                | Dop3 (D2 like) agonist                                                            | [79]            |
| Domperidone    | D2, D3 receptor antagonist                                                          | Dop3 (D2 like) agonist                                                            | [80]            |
| Bromperidine   | D2, D3, 5HT receptor agonist, α2-adrenergic, D1 receptor agonist, inactivates dopamine D4 and 5-HT7 receptors | Dop3 (D2 like) agonist                                                            | [81]            |
| Serotonin (5HT) | 5HT agonist                                                                       | 5HT1, 2,7 agonist, Dop3 (D2 like) agonant                                          | [55,61,63,66]   |
| 5-MeO-DMT      | 5HT agonist                                                                        | 5HT2 agonist                                                                      | [63,84]         |
| Ketanserin     | 5HT antagonist                                                                     | ND                                                                                 | [85]            |
| Citalopram     | SERT inhibitor                                                                     | ND                                                                                 | [86]            |

ND = not determined.
melanisation post-oviposition and this was rescued by the addition of DOPA, suggesting that at that stage melanisation depends highly on dopamine availability (Figure 4F).

**Locomotor activity is severely disturbed by activation of the adrenergic system and inhibition of the dopaminergic and serotonergic system.** Normally, following anaesthesia with a brief pulse of CO₂, mosquitoes will require several minutes to regain posture and be able to fly again. However, in our oviposition assays we observed that upon injection of tyramine, clonidine, clenbuterol and tyramine + DOPA females required a longer recovery period post-anaesthesia of more than 2 h. In addition tyramine-injected females showed leg tremors and flight inability during that time and most of the tyramine + DOPA injected females died. This suggested that we were able to interfere in neuromuscular transmission which is required for mosquito locomotor behaviour. This is in concordance with previous studies that have shown that biogenic amines can modulate this function in other insect species. To ascertain the relative contribution of the adrenergic and dopaminergic pathways to locomotor activity we injected female mosquitoes with a panel of agonists/antagonists that are known to interfere in these pathways in the human nervous system and measured the post-immobilisation recovery (PIR) time as a proxy for regain of locomotive function. We then classified the outcome in 3 groups (Figure 5A): PIR not statistically different from PBS-injected controls (green line); significant longer PIR but >50% recovery within 3 h (orange line); significant longer PIR but <50% recovery within 3 h (red line). Our results showed that in general drug-mediated dopamine receptor antagonists (Sch-39166, amitriptyline), α-adrenergic (tyramine, clonidine, prazosin) and β-adrenergic (sotalol, acebutolol, dobutamine and clenbuterol) agonists and antagonists prolonged PIR significantly. The antagonist yohimbine was able to reverse the negative effect of tyramine on the locomotory behaviour. Although amitriptyline has been recently identified as dopamine receptor (Dop2) antagonist in *Ae. aegypti*, this compound has been characterised in humans mainly as inhibitor of the re-uptake of serotonin and noradrenaline. In order to test whether the prolonged PIR seen in amitriptyline-injected females could be also caused by interference in the serotonergic system we tested other serotonergic agonists and antagonists. Indeed, compounds that are known to inhibit serotonin receptors (ketanserin) or serotonin re-uptake (citalopram) in human nervous system caused a significant increase in PIR. Citalopram and combinatory injections of amitriptyline with dopamine showed the strongest effect of all tested aminergic molecules even, in the case of citalopram, at concentrations as low as 0.25 mM (data not shown). The fact that dopamine co-injection did not rescue the effect of amitriptyline suggests that amitriptyline affects non-dopaminergic signalling in *An. gambiae* mosquitoes. Moreover, dopamine actually prolonged the PIR in coinjection with amitriptyline as well as with the α2 agonists tyramine and clonidine. Potentially these effects could be caused either by an increased imbalance between the different aminergic systems or by dopamine binding also to the respective adrenergic or serotonergic receptors thereby aggravating the effect caused by tyramine, clonidine or amitriptyline alone. The latter hypothesis is supported by studies which found that dopamine is an Octα2R (OA3), TyR1 and TyR3 agonist in the insect nervous system. However, it also seems plausible that a balance of these systems is essential as often different biogenic amines have opposing effects on behaviours such as egg laying and locomotion in insects.

Most of the females which were not able to resume flying within 3 h died within 24 h (Figure 5B).

Together, these results showed that disruption of the β-adrenergic signalling, activation of the α2-adrenergic system, inactivation of the α1-adrenergic system, inhibition of the D1-aminergic receptor or...
Figure 4 | Premature egg melanisation phenotype mediated by tyramine. (a) Ovary dissection of PBS (control) and tyramine-injected females 3 days post-bloodmeal. (b) Representative examples of eggs dissected from female ovaries ~24 h after aminegenic compound injection. (c) Mean ± SEM melanisation ratio of egg batches dissected from ovaries of 31–35 injected females from 3 repeats. (d) Mean ± SEM number of eggs laid by females following injection of tyramine alone or in combination with other compounds (N=10 per experiment, minimum of 3 experiments, Student’s t-test, in red p<0.05). (e) Proportion of females that survived 24 h post-injection with compounds (N=10, minimum of 3 repeats, Student’s t-test, in red p<0.05). (f) Mean ± SEM melanisation ratio of egg batches laid by injected females (N=10 per experiment, 3 experiments, Student’s t-test, in red p<0.05).
Figure 5 | Effect of aminergic compounds on flying ability and survival. (a) Flying ability of females in response to injection of aminergic compounds at various concentrations after CO2 knockdown (N=15). Effects were grouped in 3 classes: in green - no effect; in orange- significant effect, but 50% of females recovered within 3 h; in red- significant effect, recovery lower than 50% within 3 h. The experiment was performed in a minimum of 3 independent repeats. Curves were analysed by non-linear regression (one phase association, constraint: plateau level lower than 0.7, extra sum of squares F-test, p<0.05 is significant). ND-not determined (b) Survival rate of 15 females injected with compounds that caused significant effects on flight recovery (N=15 per experiment, 3 repeats, Student’s t-test, p<0.05 is significant).
muscles, octopamine has been shown to cause reduced muscle con-

Ae. aegypti fever mosquito showed adult toxicity for their effect in larval stages. The most 
detailed analysis of the locomotor behaviour following injection showed that interferences in the different amineergic systems caused 
distinct behavioural phenotypes (Supplementary Figure S1).

**Amitriptyline, citalopram and tyramine are toxic for mosquito larvae.** Finally, we tested a range of the same compounds that showed adult toxicity for their effect in larval stages. The most 
potent of these compounds was amitriptyline, which at a 
concentration of 0.4 mM killed more than 90% of larvae within 24 h (Figure 6A). This is comparable to its activity in the yellow fever mosquito Ae. aegypti. Tyramine and citalopram were able to 
kill larvae but required significantly higher concentrations (40 mM) (Figure 6B).

**Discussion**

Biogenic amines are responsible for the regulation of major physiological processes. They have been extensively studied in humans and 
recent progress has been made to evaluate their function in insects. We recently showed that the knockdown of a key enzyme involved in 
phenylalanine/tyrosine metabolism caused reduced fertility in the malaria mosquito. Because this pathway also regulates the synthesis 
of 3 of the 5 insect biogenic amines we investigated how amines and 
compounds bind to different adrenergic receptors, this would explain the lack of premature egg melanisation observed upon octo-
pamine and clonidine injection. The regulation of melanin production 
via adrenergic compounds has been also recently observed in 

ticks whereby injection of the α2-adrenergic agonist guanabenz acet-
ate caused whole body melanisation. In our case, the melanisation 
induced by tyramine seemed to be limited to the eggs. Given these 
results, we therefore propose that oviposition is regulated via the 

tyraminergic and tyraminergic system, but that egg melanisation 
is mediated via the tyraminergic/α2 adrenergic system in An. gambiae mosquitoes.

In line with tyraminergic regulation of egg chorion melanisation the injection of the adrenergic antagonists prazosin and yohimbine 
reduced the level of premelanalisation of eggs. We could not confirm 
this for the dopamine synthesis inhibitor carbidopa, although we 
previously showed that its injection can cause reduced melanisation of 



![Figure 6](https://www.nature.com/scientificreports/)

**Figure 6** | Larval survival in the presence of dissolved tyramine, citalopram and amitriptyline. (a) In 5 repeats the survival of 10 larvae per 
treatment (final concentration: 40 mM, 1 mM, 400 uM, 100 uM) was 
monitored and compared to the PBS control over a period of 24 h. 
(Student’s t-test, p<0.05 is significant). (b) Larval survival rate (N=10) 
within 6 h after rearing in 40 mM compound solution.

serotonin transport/receptors can each cause defects in the female 
locomotor behaviour which can result further in adult death. A 
detailed analysis of the locomotor behaviour following injection showed that interferences in the different amineergic systems caused 
distinct behavioural phenotypes (Supplementary Figure S1).

We finally tested whether adrenergic, dopaminergic and serotonon- 
ergic compounds affect the adult locomotor behaviour, which similar to female reproduction is obviously a determining factor for 
mosquito vectorial capacity. Interference in the adrenergic system 
by α2-agonists, α1-antagonists, β-agonists and antagonists led to 
reduced flight recovery. The β2-agonist clenbuterol was particularly effective, but over 40% of females recovered within 24 h. In contrast 
this to the, at the same concentration inhibition of the dopaminergic and 
serotonin transport/receptor system by amitriptyline and citalopram 
led to a severe effect on flight recovery but also increased adult 
mortality to 80–100 percent within 24 h. Interestingly, in combina-
tion with dopamine, which in itself did not have an effect on loco-
motor activity or survival, the inhibitory effect of tyramine, clonidine 
and amitriptyline was accentuated. This could have been caused by 
dopamine binding to the respective adrenergic or serotonergic recep-
tors or imbalances between the different amineergic systems. Dop-
amine has been found to bind various octopamine and tyramine 
receptors in insects and activation of different amineergic systems can 
cause opposing effects, highlighting the importance of a critical balance between these systems to maintain body function. Combinatory 

dobservable insecticides that activate one system but inhibit another could be 
therefore more effective in killing mosquitoes. Our compound con-

centration used were comparable to other studies validating dopa-
mic compounds. It remains to be seen how these correlate to 
concentrations that would be required in aerosol for our compounds to 
affet processes, such as oviposition, locomotion or respiration.

We finally tested whether adrenergic, dopaminergic and seroton- 
nergic molecules would be effective larvicides. We found that
although tyramine, citalopram and amitriptyline were able to significantly reduce larval survival rapidly, the latter was the most toxic and although tyramine, citalopram and amitriptyline were able to significantly reduce larval survival rapidly, the latter was the most toxic and

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Acknowledgments

This Research was funded by grants from European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreements N° 242095 (EviMalaR) and N° 228421 (INFRAVEC) and the Foundation for the National Institutes of Health through the Vector-Based Control of Transmission: Discovery Research (VCTR) program of the Grand Challenges in Global Health initiative. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We also would like to thank Ann Hall for rearing of the mosquitoes.

Author contributions

S.F. and E.R. performed experiments. S.F. analysed data and prepared figures. S.F., T.N. and A.C. wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Fuchs, S., Rende, E., Crisanti, A. & Nolan, T. Disruption of aminegeric signalling reveals novel compounds with distinct inhibitory effects on mosquito reproduction, locomotor function and survival. Sci. Rep. 4, 5526; DOI:10.1038/srep05526 (2014).

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